IMMUNOSTIMULATORY OLIGONUCLEOTIDES

RELATED APPLICATIONS

This application is a continuation in part of U.S. Serial No. 08/738,652, filed October 30, 1996, pending, which is a continuation-in-part of U.S. Patent Application serial number 08/386,063, filed February 7, 1995 currently pending, which is a continuation-in-part of U.S. Patent Application 08/276,358, filed July 15, 1994 which is now abandoned, each of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

The work resulting in this invention was supported in part by National Institute of Health Grant No. R29-AR42556-01. The U.S. Government may therefore be entitled to certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated CpG dinucleotide which are immunostimulatory.

BACKGROUND OF THE INVENTION

DNA binds to cell membranes and is internalized

In the 1970's, several investigators reported the binding of high molecular weight DNA to cell membranes (Lerner, R.A., W. Meinke, and D.A. Goldstein. 1971. "Membrane-associated DNA in the cytoplasm of diploid human lymphocytes". *Proc. Natl. Acad. Sci. USA 68:1212*; Agrawal, S.K., R.W. Wagner, P.K. McAllister, and B. Rosenberg. 1975. "Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum-pyrimidine complexes by electron microscopy". *Proc. Natl. Acad. Sci. USA 72:928*). In 1985, Bennett et al. presented the first evidence that DNA binding to lymphocytes is similar to a ligand receptor interaction: binding is saturable, competitive, and leads to DNA endocytosis and degradation into oligonucleotides (Bennett, R.M., G.T. Gabor, and M.M. Merritt. 1985. "DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA". *J. Clin. Invest. 76:2182*). Like DNA, oligodeoxyribonucleotides (ODNs) are able to enter cells in a saturable, sequence independent, and temperature and energy dependent fashion (reviewed in Jaroszewski, J.W., and J.S. Cohen. 1991. "Cellular uptake of antisense oligodeoxynucleotides". *Advanced Drug*

Delivery Reviews 6:235; Akhtar, S., Y. Shoji, and R.L. Juliano. 1992. "Pharmaceutical aspects of the biological stability and membrane transport characteristics of antisense oligonucleotides". In: Gene Regulation: Biology of Antisense RNA and DNA. R.P. Erickson, and J.G. Izant, eds. Raven Press, Ltd. New York, *pp. 133*; and Zhao, Q., T. Waldschmidt, E. Fisher, C.J. Herrera, and A.M. Krieg., 1994. "Stage specific oligonucleotide uptake in murine bone marrow B cell precursors". *Blood,* 84:3660). No receptor for DNA or ODN uptake has yet been cloned, and it is not yet clear whether ODN binding and cell uptake occurs through the same or a different mechanism from that of high molecular weight DNA.

Lymphocyte ODN uptake has been shown to be regulated by cell activation. Spleen cells stimulated with the B cell mitogen LPS had dramatically enhanced ODN uptake in the B cell population, while spleen cells treated with the T cell mitogen Con A showed enhanced ODN uptake by T but not B cells (Krieg, A.M., F. Gmelig-Meyling, M.F. Gourley, W.J. Kisch, L.A. Chrisey, and A.D. Steinberg. 1991. "Uptake of oligodeoxyribonucleotides by lymphoid cells is heterogeneous and inducible". *Antisense Research and Development 1:161*).

Immune effects of nucleic acids

Several polynucleotides have been extensively evaluated as biological response modifiers. Perhaps the best example is poly (I,C) which is a potent inducer of IFN production as well as a macrophage activator and inducer of NK activity (Talmadge, J.E., J. Adams, H. Phillips, M. Collins, B. Lenz, M. Schneider, E. Schlick, R. Ruffmann, R.H. Wiltrout, and M.A. Chirigos. 1985. "Immunomodulatory effects in mice of polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose". *Cancer Res. 45:1058*; Wiltrout, R.H., R.R. Salup, T.A. Twilley, and J.E. Talmadge. 1985. "Immunomodulation of natural killer activity by polyribonucleotides". *J. Biol. Resp. Mod. 4:512*; Krown, S.E. 1986. "Interferons and interferon inducers in cancer treatment". *Sem. Oncol. 13:207*; and Ewel, C.H., S.J. Urba, W.C. Kopp, J.W. Smith II, R.G. Steis, J.L. Rossio, D.L. Longo, M.J. Jones, W.G. Alvord, C.M. Pinsky, J.M. Beveridge, K.L. McNitt, and S.P. Creekmore. 1992. "Polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose in combination with interleukin-2 in patients with cancer: clinical and immunological effects". *Canc. Res. 52:3005*). It appears that this murine NK activation may be due solely to induction of IFN-β secretion (Ishikawa, R., and C.A. Biron. 1993. "IFN induction and associated changes in splenic leukocyte distribution". *J. Immunol. 150:3713*). This activation was specific for the ribose sugar since deoxyribose was ineffective. Its potent in vitro antitumor activity led to several clinical trials using poly (I,C) complexed with poly-L-lysine and carboxymethylcellulose (to reduce degradation by RNAse) (Talmadge, J.E., et al., 1985. cited *supra*; Wiltrout, R.H., et al., 1985. cited *supra*); Krown, S.E., 1986. cited

supra); and Ewel, C.H., et al., 1992. cited *supra*). Unfortunately, toxic side effects have thus far prevented poly (I,C) from becoming a useful therapeutic agent.

Guanine ribonucleotides substituted at the C8 position with either a bromine or a thiol group are B cell mitogens and may replace "B cell differentiation factors" (Feldbush, T.L., and Z.K. Ballas. 1985. "Lymphokine-like activity of 8-mercaptoguanosine: induction of T and B cell differentiation". *J. Immunol. 134:3204*; and Goodman, M.G. 1986. "Mechanism of synergy between T cell signals and C8-substituted guanine nucleosides in humoral immunity: B lymphotropic cytokines induce responsiveness to 8-mercaptoguanosine". *J. Immunol. 136:3335*). 8-mercaptoguanosine and 8-bromoguanosine also can substitute for the cytokine requirement for the generation of MHC restricted CTL (Feldbush, T.L., 1985. cited *supra*), augment murine NK activity (Koo, G.C., M.E. Jewell, C.L. Manyak, N.H. Sigal, and L.S. Wicker. 1988. "Activation of murine natural killer cells and macrophages by 8-bromoguanosine". *J. Immunol. 140:3249*), and synergize with IL-2 in inducing murine LAK generation (Thompson, R.A., and Z.K. Ballas. 1990. "Lymphokine-activated killer (LAK) cells. V. 8-Mercaptoguanosine as an IL-2-sparing agent in LAK generation". *J. Immunol. 145:3524*). The NK and LAK augmenting activities of these C8-substituted guanosines appear to be due to their induction of IFN (Thompson, R.A., et al. 1990. cited *supra*). Recently, a 5' triphosphorylated thymidine produced by a mycobacterium was found to be mitogenic for a subset of human γδ T cells (Constant, P., F. Davodeau, M.-A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J.-J. Fournie. 1994. "Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands" *Science 264:267*). This report indicated the possibility that the immune system may have evolved ways to preferentially respond to microbial nucleic acids.

Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For example, Bell et al. reported that nucleosomal protein-DNA complexes (but not naked DNA) in spleen cell supernatants caused B cell proliferation and immunoglobulin secretion (Bell, D.A., B. Morrison, and P. VandenBygaart. 1990. "Immunogenic DNA-related factors". *J. Clin. Invest. 85:1487*). In other cases, naked DNA has been reported to have immune effects. For example, Messina et al. have recently reported that 260 to 800 bp fragments of poly (dG)•(dC) and poly (dG•dC) were mitogenic for B cells (Messina, J.P., G.S. Gilkeson, and D.S. Pisetsky. 1993. "The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens". *Cell. Immunol.147:148*). Tokunaga, et al. have reported that dG•dC induces γ-IFN and NK activity (Tokunaga, S. Yamamoto, and K. Namba. 1988. "A synthetic single-stranded DNA, poly(dG,dC), induces interferon-α/β and -γ, augments natural killer activity, and suppresses tumor growth" *Jpn. J. Cancer Res. 79:682*). Aside from such artificial homopolymer sequences, Pisetsky et al. reported that pure mammalian DNA has no

detectable immune effects, but that DNA from certain bacteria induces B cell activation and immunoglobulin secretion (Messina, J.P., G.S. Gilkeson, and D.S. Pisetsky. 1991. "Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA". *J. Immunol. 147:1759*). Assuming that these data did not result from some unusual contaminant, these studies suggested that a particular structure or other characteristic of bacterial DNA renders it capable of triggering B cell activation. Investigations of mycobacterial DNA sequences have demonstrated that ODN which contain certain palindrome sequences can activate NK cells (Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. "Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity". *J. Immunol. 148:4072;* Kuramoto, E., O. Yano, Y. Kimura, M. Baba, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1992. "Oligonucleotide sequences required for natural killer cell activation". *Jpn. J. Cancer Res. 83:1128*).

Several phosphorothioate modified ODN have been reported to induce in vitro or in vivo B cell stimulation (Tanaka, T., C.C. Chu, and W.E. Paul. 1992. "An antisense oligonucleotide complementary to a sequence in Iγ2b increases γ2b germline transcripts, stimulates B cell DNA synthesis, and inhibits immunoglobulin secretion". *J. Exp. Med. 175:597*; Branda, R.F. , A.L. Moore, L. Mathews, J.J. McCormack, and G. Zon. 1993. "Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1". *Biochem. Pharmacol. 45:2037*; McIntyre, K.W., K. Lombard-Gillooly, J.R. Perez, C. Kunsch, U.M. Sarmiento, J.D. Larigan, K.T. Landreth, and R. Narayanan. 1993. "A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-κβ T65 causes sequence-specific immune stimulation". *Antisense Res. Develop. 3:309*; and Pisetsky, D.S., and C.F. Reich. 1993. "Stimulation of murine lymphocyte proliferation by a phosphorothioate oligonucleotide with antisense activity for herpes simplex virus". *Life Sciences 54:101*). These reports do not suggest a common structural motif or sequence element in these ODN that might explain their effects.

The CREB/ATF family of transcription factors and their role in replication

The cAMP response element binding protein (CREB) and activating transcription factor (ATF) or CREB/ATF family of transcription factors is a ubiquitously expressed class of transcription factors of which 11 members have so far been cloned (reviewed in de Groot, R.P., and P. Sassone-Corsi: "Hormonal control of gene expression: Multiplicity and versatility of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators". *Mol. Endocrin. 7*:145, 1993; Lee, K.A.W., and N. Masson: "Transcriptional regulation by CREB and its relatives". *Biochim. Biophys. Acta* 1174:221, 1993.). They all belong to the basic region/leucine zipper (bZip) class of proteins. All cells appear to express one or more CREB/ATF proteins, but the members expressed and the regulation of mRNA splicing

appear to be tissue-specific. Differential splicing of activation domains can determine whether a particular CREB/ATF protein will be a transcriptional inhibitor or activator. Many CREB/ATF proteins activate viral transcription, but some splicing variants which lack the activation domain are inhibitory. CREB/ATF proteins can bind DNA as homo- or hetero-dimers through the cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the CpG is methylated) (Iguchi-Ariga, S.M.M., and W. Schaffner: "CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation". *Genes & Develop. 3*:612, 1989.).

The transcriptional activity of the CRE is increased during B cell activation (Xie, H. T.C. Chiles, and T.L. Rothstein: "Induction of CREB activity via the surface Ig receptor of B cells". *J. Immunol. 151*:880, 1993.). CREB/ATF proteins appear to regulate the expression of multiple genes through the CRE including immunologically important genes such as fos, jun B, Rb-1, IL-6, IL-1 (Tsukada, J., K. Saito, W.R. Waterman, A.C. Webb, and P.E. Auron: "Transcription factors NF-IL6 and CREB recognize a common essential site in the human prointerleukin 1β gene". *Mol. Cell. Biol. 14*:7285, 1994; Gray, G.D., O.M. Hernandez, D. Hebel, M. Root, J.M. Pow-Sang, and E. Wickstrom: "Antisense DNA inhibition of tumor growth induced by c-Ha-ras oncogene in nude mice". *Cancer Res. 53*:577, 1993), IFN-β (Du, W., and T. Maniatis: "An ATF/CREB binding site protein is required for virus induction of the human interferon B gene". *Proc. Natl. Acad. Sci. USA 89*:2150, 1992), TGF-β1 (Asiedu, C.K., L. Scott, R.K. Assoian, M. Ehrlich: "Binding of AP-1/CREB proteins and of MDBP to contiguous sites downstream of the human TGF-B1 gene". *Biochim. Biophys. Acta 1219*:55, 1994.), TGF-β2, class II MHC (Cox, P.M., and C.R. Goding: "An ATF/CREB binding motif is required for aberrant constitutive expression of the MHC class II DRa promoter and activation by SV40 T-antigen". *Nucl. Acids Res. 20*:4881, 1992.), E-selectin, GM-CSF, CD-8α, the germline Igα constant region gene, the TCR Vβ gene, and the proliferating cell nuclear antigen (Huang, D., P.M. Shipman-Appasamy, D.J. Orten, S.H. Hinrichs, and M.B. Prystowsky: "Promoter activity of the proliferating-cell nuclear antigen gene is associated with inducible CRE-binding proteins in interleukin 2-stimulated T lymphocytes". *Mol. Cell. Biol. 14*:4233, 1994.). In addition to activation through the cAMP pathway, CREB can also mediate transcriptional responses to changes in intracellular Ca^{++} concentration (Sheng, M., G. McFadden, and M.E. Greenberg: "Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB". *Neuron 4*:571, 1990).

The role of protein-protein interactions in transcriptional activation by CREB/ATF proteins appears to be extremely important. There are several published studies reporting direct or indirect interactions between NFKB proteins and CREB/ATF proteins (Whitley, et.

al., (1994) *Mol. & Cell. Biol.* 14:6464; Cogswell, et al., (1994) *J. Immun.* 153:712; Hines, et al., (1993) *Oncogene* 8:3189; and Du, et al., (1993) *Cell* 74:887. Activation of CREB through the cyclic AMP pathway requires protein kinase A (PKA), which phosphorylates $CREB^{341}$ on ser^{133} and allows it to bind to a recently cloned protein, CBP (Kwok, R.P.S., J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bachinger, R.G. Brennan, S.G.E. Roberts, M.R. Green, and R.H. Goodman: "Nuclear protein CBP is a coactivator for the transcription factor CREB". *Nature 370*:223, 1994; Arias, J., A.S. Alberts, P. Brindle, F.X. Claret, T. Smea, M. Karin, J. Feramisco, and M. Montminy: "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor". *Nature 370*:226, 1994.). CBP in turn interacts with the basal transcription factor TFIIB causing increased transcription. CREB also has been reported to interact with dTAFII 110, a TATA binding protein-associated factor whose binding may regulate transcription (Ferreri, K., G. Gill, and M. Montminy: "The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex". *Proc. Natl. Acad. Sci. USA 91*:1210, 1994.). In addition to these interactions, CREB/ATF proteins can specifically bind multiple other nuclear factors (Hoeffler, J.P., J.W. Lustbader, and C.-Y. Chen: "Identification of multiple nuclear factors that interact with cyclic adenosine 3',5'-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions". *Mol. Endocrinol. 5*:256, 1991) but the biologic significance of most of these interactions is unknown. CREB is normally thought to bind DNA either as a homodimer or as a heterodimer with several other proteins. Surprisingly, CREB monomers constitutively activate transcription (Krajewski, W.; and K.A.W. Lee: "A monomeric derivative of the cellular transcription factor CREB functions as a constitutive activator". *Mol. Cell. Biol. 14*:7204, 1994.).

Aside from their critical role in regulating cellular transcription, it has recently been shown that CREB/ATF proteins are subverted by some infectious viruses and retroviruses, which require them for viral replication. For example, the cytomegalovirus immediate early promoter, one of the strongest known mammalian promoters, contains eleven copies of the CRE which are essential for promoter function (Chang, Y.-N., S. Crawford, J. Stall, D.R. Rawlins, K.-T. Jeang, and G.S. Hayward: "The palindromic series I repeats in the simian cytomegalovirus major immediate-early promoter behave as both strong basal enhancers and cyclic AMP response elements". *J. Virol. 64*:264, 1990). At least some of the transcriptional activating effects of the adenovirus E1A protein, which induces many promoters, are due to its binding to the DNA binding domain of the CREB/ATF protein, ATF-2, which mediates E1A inducible transcription activation (Liu, F., and M.R. Green: "Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains". *Nature 368*:520, 1994). It has also been suggested that E1A binds to the CREB-binding protein, CBP (Arany, Z., W.R. Sellers, D.M. Livingston, and R. Eckner: "E1A-associated p300 and

CREB-associated CBP belong to a conserved family of coactivators". *Cell 77*:799, 1994). Human T lymphotropic virus-I (HTLV-1), the retrovirus which causes human T cell leukemia and tropical spastic paresis, also requires CREB/ATF proteins for replication. In this case, the retrovirus produces a protein, Tax, which binds to CREB/ATF proteins and redirects them from their normal cellular binding sites to different DNA sequences (flanked by G- and C-rich sequences) present within the HTLV transcriptional enhancer (Paca-Uccaralertkun, S., L.-J. Zhao, N. Adya, J.V. Cross, B.R. Cullen, I.M. Boros, and C.-Z. Giam: "In vitro selection of DNA elements highly responsive to the human T-cell lymphotropic virus type I transcriptional activator, Tax". *Mol. Cell. Biol. 14*:456, 1994; Adya, N., L.-J. Zhao, W. Huang, I. Boros, and C.-Z. Giam: "Expansion of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282-284 near the conserved DNA-binding domain of CREB". *Proc. Natl. Acad. Sci. USA 91*:5642, 1994).

SUMMARY OF THE INVENTION

The instant invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (CpG) dinucleotides activate lymphocytes and other immune cells in a subject and stimulate a subject's immune response (e.g. by inducing monocytic cells and other cells to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel immunostimulatory nucleic acid compositions.

The invention in one aspect is a composition of an oligonucleotide comprising 5' $Y_1N_1CGN_2Y_2$ 3' wherein Y_1 and Y_2 are, independent of one another, nucleic acid molecules having between 1 and 10 nucleotides, and wherein Y_1 includes at least one modified internucleotide linkage and Y_2 includes at least one modified internucleotide linkage and wherein N_1 and N_2 are nucleic acid molecules, each independent of one another having between 0 and 20 nucleotides, but wherein N_1CGN_2 has at least 6 nucleotides in total and wherein the nucleotides of N_1CGN_2 have a phosphodiester backbone. In some embodiments Y_1 and/or Y_2 have between 3 and 8 nucleotides. In other embodiments Y_1 and/or Y_2 are comprised of at least three Gs, at least four Gs, least seven Gs, or all Gs. In other embodiments Y_1 and/or Y_2 are selected from the group consisting of TCGTCG (SEQ ID NO:65), TCGTCGT (SEQ ID NO:66), and TCGTCGTT (SEQ ID NO:67). N_1 and N_2, in other embodiments have between 3 and 8 nucleotides.

The center nucleotides (N_1CGN_2) of the formula $Y_1N_1CGN_2Y_2$ have phosphodiester internucleotide linkages and Y_1 and Y_2 have at least one modified internucleotide linkage. In

some embodiments Y_1 and/or Y_2 have at least two modified internucleotide linkages. In other embodiments Y_1 and/or Y_2 have between two and five modified internucleotide linkages. In yet other embodiments Y_1 has two modified internucleotide linkages and Y_2 has five modified internucleotide linkages or Y_1 has five modified internucleotide linkages and Y_2 has two modified internucleotide linkages. The modified internucleotide linkage, in some embodiments is a phosphorothioate modified linkage, a phosphorodithioate modified linkage or a p-ethoxy modified linkage.

Portions of the formula $Y_1N_1CGN_2Y_2$ may optionally form a palindrome. Thus, in some embodiments the nucleotides of N_1CGN_2 form a palindrome. In yet other embodiments the nucleotides of N_1CGN_2 do not form a palindrome.

In one preferred embodiment the oligonucleotide has a sequence of nucleotides GGGGTCAACGTTGAGGGGGG (SEQ ID NO:12).

The composition may optionally include a pharmaceutical carrier and/or be formulated in a delivery device. In some embodiments the delivery device is selected from the group consisting of cationic lipids, cell permeating proteins, and sustained release devices. In one preferred embodiment the sustained release device is a biodegradable polymer. In another embodiment the sustained release device is a microparticle or microsphere.

In another aspect the compositions described above also include an immunostimulatory nucleic acid having an unmethylated CG dinucleotide, wherein the immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a different sequence than the oligonucleotide comprising 5' $Y_1N_1CGN_2Y_2$ 3'.

In some embodiments the immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a completely phosphodiester backbone and in other embodiments the immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a modified backbone, which optionally may have internucleotide linkages selected from the group consisting of phosphorothioate, phosphorodithioate, and -p-ethoxy.

In one embodiment immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a formula comprising: 5' $X_1X_2CGX_3X_4$ 3' wherein X_1, X_2, X_3 and X_4 are nucleotides. In other embodiments the immunostimulatory nucleic acid sequence includes at least the following formula: 5' $TCNTX_1X_2CGX_3X_4$ 3' wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides, wherein at least one nucleotide has a modified

internucleotide linkage, and wherein the nucleic acid has less than or equal to 100 nucleotides. According to some embodiments X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or GpT. In a preferred embodiment X_1X_2 are GpA and X_3X_4 are TpT.

In another embodiment the immunostimulatory nucleic acid sequence includes at least one of the following sequences: ATCGACTCTCGAGCGTTCTC (SEQ ID NO:18); TCCATGTCGGTCCTGCTGAT (SEQ ID NO:35); TCCATGTCGGTZCTGATGCT (SEQ ID NO:34); ATCGACTCTCGAGCGTTZTC (SEQ ID NO:21); TCCATGTCGGTCCTGATGCT (SEQ ID NO:31); GGGGTCAACGTTGAGGGGGG (SEQ ID NO:12); TCCATGACGGTCCTGATGCT (SEQ ID NO:40); TCCATGGCGGTCCTGATGCT (SEQ ID NO:39); TCCATGACGTTCCTGATGCT (SEQ ID NO:44); TCCATGTCGTTCCTGATGCT (SEQ ID NO:43); GGGGTCAGTCTTGACGGGG (SEQ ID NO:50); TCCATGTCGCTCCTGATGCT (SEQ ID NO:42); TCCATGTCGATCCTGATGCT (SEQ ID NO:41); TCCATGCCGGTCCTGATGCT (SEQ ID NO:38); TCCATAACGTTCCTGATGCT (SEQ ID NO:45); TCCATGACGTCCCTGATGCT (SEQ ID NO:46); TCCATCACGTGCCTGATGCT (SEQ ID NO:47); and TCCATGACGTTCCTGACGTT (SEQ ID No.10).

In another aspect the invention relates to a pharmaceutical composition including at least two oligonucleotides of the invention, wherein the at least two oligonucleotides have different sequences from one another and a pharmaceutically acceptable carrier.

According to another aspect of the invention, a method for activating an NK cell is provided. The method involves contacting an NK cell with any of the compositions of the invention in order to activate the NK cell.

In another aspect the invention is a method for activating a dendritic cell by contacting a dendritic cell with any of the compositions of the invention in order to activate the dendritic cell.

In yet another aspect the invention is a method for activating a lymphocyte by contacting a lymphocyte with any of the compositions of the invention in order to activate the lymphocyte.

A method for treating or preventing a cancer is also provided according to an aspect of the invention. The method involves administering to a subject having or at risk of having cancer, an effective amount for treating or preventing the cancer of any of the compositions of the invention. In one embodiment the cancer is selected from the group consisting of hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, and colon carcinoma.

A vaccine formulation is provided according to another aspect of the invention. The vaccine includes any of the compositions of the invention in combination with an antigen.

In other aspects, the invention is a combination composition or method. In these aspects, the oligonucleotide is combined with another therapeutic such as an anti-cancer medicament or an antibody.

According to another aspect of the invention a method for treating or preventing a viral or retroviral infection is provided. The method involves administering to a subject having or at risk of having a viral or retroviral infection, an effective amount for treating or preventing the viral or retroviral infection of any of the compositions of the invention. In some embodiments the virus is caused by a hepatitis virus, HIV, hepatitis B, hepatitis C, herpes virus, retroviridae, herpesviridae, orthomyxiviridae, or papillomavirus.

In some embodiments, the subject is a human and in other embodiments, the subject is a non-human subject.

A method for treating or preventing a bacterial infection is provided according to another aspect of the invention. The method involves administering to a subject having or at risk of having a bacterial infection, an effective amount for treating or preventing the bacterial infection of any of the compositions of the invention. In one embodiment the bacterial infection is due to a bacteria such as helicobacter pylori, staphyloccocus, streptococcus, haemophilus, enterobacter, clostridium, campylobacter, or E.coli.

In another aspect the invention is a method for treating or preventing a parasite infection by administering to a subject having or at risk of having a parasite infection, an effective amount for treating or preventing the parasite infection of any of the compositions of the invention. In one embodiment the parasite infection is due to plasmodium falciparum or toxoplasma gondii.

In yet another aspect, the invention is a method for treating or preventing asthma, by administering to a subject having or at risk of having asthma, an effective amount for treating or preventing the asthma of any of the compositions of the invention. In one embodiment the asthma is allergic asthma.

In another aspect the invention relates to a method for treating or preventing allergy. The method involves administering to a subject having or at risk of having allergy, an effective amount for treating or preventing the allergy of any of the compositions of the invention.

A method for treating or preventing an immune deficiency is provided according to another aspect of the invention. The method involves administering to a subject having or at risk of an immune deficiency, an effective amount for treating or preventing the immune deficiency of any of the compositions of the invention.

In another aspect the invention relates to a method for inducing a TH1 immune response by administering to a subject any of the compositions of the invention in an effective amount to produce a TH1 immune response.

In one embodiment the methods of the invention involve administering an oligonucleotide of formula 5' $Y_1N_1CGN_2Y_2$ 3' and an immunostimulatory nucleic acid having an unmethylated CG dinucleotide.

Each of the limitations of the invention can encompass various embodiments of the invention. It is therefore, anticipated that each of the limitations of the invention involving any one element or combination of elements can be included in each aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C are graphs plotting dose-dependent IL-6 production in response to various DNA sequences in T cell depleted spleen cell cultures. **A.** *E. coli* DNA (●) and calf thymus DNA (■) sequences and LPS (at 10x the concentration of *E. coli* and calf thymus DNA) (◆). **B.** Control phosphodiester oligodeoxynucleotide (ODN) $5'$ATGGAAGGTCCAGTGTTCTC$3'$ (SEQ ID No: 1) (■) and two phosphodiester CpG ODN $5'$ATCGACCTACGTGCGTTCTC$3'$ (SEQ ID No: 2) (◆) and $5'$TCCATAACGTTCCTGATGCT$3'$ (SEQ ID No: 3) (●). **C.** Control phosphorothioate ODN $5'$GCTAGATGTTAGCGT$3'$ (SEQ ID No: 4) (■) and two phosphorothioate CpG ODN $5'$GAGAACGTCGACCTTCGAT$3'$ (SEQ ID No: 5) (◆) and $5'$GCATGACGTTGAGCT$3'$ (SEQ ID No: 6) ( ). Data present the mean ± standard deviation of triplicates.

Figure 2 is a graph plotting IL-6 production induced by CpG DNA *in vivo* as determined 1-8 hrs after injection. Data represent the mean from duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were injected iv. with 100 µl of PBS (□) or 200 µg of CpG phosphorothioate ODN $5'$TCCATGACGTTCCTGATGCT$3'$ (SEQ ID No: 7) (■) or non-CpG phosphorothioate ODN $5'$TCCATGAGCTTCCTGAGTCT$3'$ (SEQ ID No: 8) (◆).

Figure 3 is an autoradiograph showing IL-6 mRNA expression as determined by reverse transcription polymerase chain reaction in liver, spleen, and thymus at various time periods after *in vivo* stimulation of BALB/c mice (two mice/group) injected iv with 100 µl of PBS, 200 µg of CpG phosphorothioate ODN $5'$TCCATGACGTTCCTGATGCT$3'$ (SEQ ID No: 7) or non-CpG phosphorothioate ODN $5'$TCCATGAGCTTCCTGAGTCT$3'$ (SEQ ID No: 8).

Figure 4A is a graph plotting dose-dependent inhibition of CpG-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with CpG ODN $5'$TCCAAGACGTTCCTGATGCT$3'$ (SEQ ID No: 9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (◆) or isotype control Ab (●) and IgM levels in culture supernatants determined by ELISA. In the absence of CpG ODN, the anti-IL-6 Ab had no effect on IgM secretion (■).

Figure 4B is a graph plotting the stimulation index of CpG-induced splenic B cells cultured with anti-IL-6 and CpG S-ODN $5'$TCCATGACGTTCCTGATGCT$3'$ (SEQ ID NO:7) (◆) or anti- IL-6 antibody only (■). Data present the mean $\pm$ standard deviation of triplicates.

Figure 5 is a bar graph plotting chloramphenicol acetyltransferase (CAT) activity in WEHI-231 cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with CpG $5'$TCCATGACGTTCCTGATGCT$3'$ (SEQ ID NO:7) or non-CpG $5'$TCCATGAGCTTCCTGAGTCT$3'$ (SEQ ID NO:8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

Figure 6 is a schematic overview of the immune effects of the immunostimulatory unmethylated CpG containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The immunostimulatory oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the immunostimulatory nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the

induction of Th1 cytokine secretion by the immunostimulatory oligonucleotides promotes the development of a cytotoxic lymphocyte response.

Figure 7 is an autoradiograph showing NFκB mRNA induction in monocytes treated with *E. coli* (EC) DNA (containing unmethylated CpG motifs), control (CT) DNA (containing no unmethylated CpG motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

Figure 8A shows the results from a flow cytometry study using mouse B cells with the dihydrorhodamine 123 dye to determine levels of reactive oxygen species. The dye only sample in Panel A of the figure shows the background level of cells positive for the dye at 28.6%. This level of reactive oxygen species was greatly increased to 80% in the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the CpG oligo (TCCATGACGTTCCTGACGTT SEQ ID NO:10) also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the CpGs were switched (TCCATGAGCTTCCTGAGTGCT SEQ ID NO:11) did not show this significant increase in the level of reactive oxygen species (Panel E).

Figure 8B shows the results from a flow cytometry study using mouse B cells in the presence of chloroquine with the dihydrorhodamine 123 dye to determine levels of reactive oxygen species. Chloroquine slightly lowers the background level of reactive oxygen species in the cells such that the untreated cells in Panel A have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with CpG DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and ionomycin (Panel E).

Figure 9 is a graph plotting lung lavage cell count over time. The graph shows that when the mice are initially injected with *Schistosoma mansoni* eggs "egg", which induces a Th2 immune response, and subsequently inhale *Schistosoma mansoni* egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given CpG oligo (SEQ ID NO:10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation of SEA (open triangles).

Figure 10 is a graph plotting lung lavage eosinophil count over time. Again, the graph shows that when the mice are initially injected with egg and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given CpG oligo (SEQ ID NO:10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation of the SEA (open triangles).

Figure 11 is a bar graph plotting the effect on the percentage of macrophage, lymphocyte, neutrophil and eosinophil cells induced by exposure to saline alone; egg, then

SEA; egg and SEQ ID NO:11, then SEA; and egg and control oligo (SEQ ID NO:11), then SEA. When the mice are treated with the control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

Figure 12 is a bar graph plotting eosinophil count in response to injection of various amounts of the protective oligo SEQ ID NO:10.

Figure 13 is a graph plotting interleukin 4 (IL-4) production (pg/ml) in mice over time in response to injection of egg, then SEA (open diamond); egg and SEQ ID NO:10, then SEA (open circle); or saline, then saline (open square). The graph shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

Figure 14 is a bar graph plotting interleukin 12 (IL-12) production (pg/ml) in mice over time in response to injection of saline; egg, then SEA; or SEQ ID NO:10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune response.

Figure 15 is a bar graph plotting interferon gamma (IFN-γ) production (pg/ml) in mice over time in response to injection of saline; egg, then saline; or SEQ ID NO:10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune response.

DETAILED DESCRIPTION

Definitions

As used herein, the following terms and phrases shall have the meanings set forth below:

An "allergen" refers to a substance that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include proteins specific to the following genuses: *Canine* (*Canis familiaris*); *Dermatophagoides* (e.g. *Dermatophagoides farinae*); *Felis* (*Felis domesticus*); *Ambrosia* (*Ambrosia artemiisfolia*; *Lolium* (e.g. *Lolium perenne* or *Lolium multiflorum*);

Cryptomeria (*Cryptomeria japonica*); *Alternaria* (*Alternaria alternata*); *Alder*; *Alnus* (*Alnus gultinosa*); *Betula* (*Betula verrucosa*); *Quercus* (*Quercus alba*); *Olea* (*Olea europa*); *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g. *Plantago lanceolata*); *Parietaria* (e.g. *Parietaria officinalis* or *Parietaria judaica*); *Blattella* (e.g. *Blattella germanica*); *Apis* (e.g. *Apis multiflorum*); *Cupressus* (e.g. *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); *Juniperus* (e.g. *Juniperus sabinoides*, *Juniperus virginiana*, *Juniperus communis* and *Juniperus ashei*); *Thuya* (e.g. *Thuya orientalis*); *Chamaecyparis* (e.g. *Chamaecyparis obtusa*); *Periplaneta* (e.g. *Periplaneta americana*); *Agropyron* (e.g. *Agropyron repens*); *Secale* (e.g. *Secale cereale*); *Triticum* (e.g. *Triticum aestivum*); *Dactylis* (e.g. *Dactylis glomerata*); *Festuca* (e.g. *Festuca elatior*); *Poa* (e.g. *Poa pratensis* or *Poa compressa*); *Avena* (e.g. *Avena sativa*); *Holcus* (e.g. *Holcus lanatus*); *Anthoxanthum* (e.g. *Anthoxanthum odoratum*); *Arrhenatherum* (e.g. *Arrhenatherum elatius*); *Agrostis* (e.g. *Agrostis alba*); *Phleum* (e.g. *Phleum pratense*); *Phalaris* (e.g. *Phalaris arundinacea*); *Paspalum* (e.g. *Paspalum notatum*); *Sorghum* (e.g. *Sorghum halepensis*); and *Bromus* (e.g. *Bromus inermis*).

An "allergy" refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include eczema, allergic rhinitis or coryza, hay fever, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions. A "subject having an allergy" is a subject that has or is at risk of developing an allergic reaction in response to an allergen.

"Asthma" - refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

An "immune system deficiency" shall mean a disease or disorder in which the subject's immune system is not functioning in normal capacity or in which it would be useful to boost a subject's immune response for example to eliminate a tumor or cancer or an infection in a subject.

Examples of infectious virus include: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g., polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g., equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus,

measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g., influenza viruses); *Bungaviridae* (e.g., Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviurses and rotaviruses); *Birnaviridae; Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses'); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g., African swine fever virus); and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatities (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

Examples of infectious bacteria include: *Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria sps (e.g. M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis, Streptococcus bovis, Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus antracis, corynebacterium diphtheriae, corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida, Bacteroides sp., Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira,* and *Actinomyces israelli.*

Examples of infectious fungi include: *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis,Chlamydia trachomatis, Candida albicans.* Other infectious organisms (i.e., protists) include: *Plasmodium falciparum* and *Toxoplasma gondii.*

The oligonucleotides are useful as prophylactics for the induction of immunity of a subject at risk of developing an infection with an infectious organism or a subject at risk of developing an allergy or cancer. A "subject at risk" as used herein is a subject who has any risk of exposure to an infection causing infectious pathogen or an allergen or of developing

cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent or allergen is found or it may be a subject who through lifestyle or medical procedures is exposed to bodily fluids which may contain infectious organisms or even any subject living in an area that an infectious organism or an allergen has been identified and is exposed directly to the infectious agent or allergen. It also may be a subject at risk of biowarfare such as military personnel or those living in areas at risk of terrorist attack. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject is exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. Subjects at risk of developing cancer include those with a genetic predisposition or previously treated for cancer, and those exposed to carcinogens such as tobacco, asbestos, and other chemical toxins or excessive sunlight and other types of radiation.

In addition to the use of the oligonucleotides for prophylactic treatment, the invention also encompasses the use of the oligonucleotides for the treatment of a subject having an infection, an allergy or a cancer.

A "subject having an infection" is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The oligonucleotide can be used with an antigen to mount an antigen specific immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body.

A "subject having a cancer" is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas. In one embodiment the cancer is hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular

lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, or colon carcinoma.

An "immunostimulatory nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing an unmethylated cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression or antibody production by) a vertebrate lymphocyte. The whole molecule may be unmethylated or part of the molecule may be unmethylated but only the C must be unmethylated. An immunostimulatory nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased immune activity.

In a preferred embodiment, the immunostimulatory nucleic acid contains a consensus mitogenic CpG motif represented by the formula:

$$5' \quad X_1CGX_2 \quad 3'$$

wherein X_1 and X_2 are nucleotides. In one embodiment X_1 is selected from the group consisting of A, G and T; and X_2 is C or T.

In a particularly preferred embodiment, immunostimulatory nucleic acid molecules having an unmethylated CG dinucleotide are between 2 to 100 base pairs in size and contain a consensus mitogenic CpG motif represented by the formula:

$$5' \quad X_1X_2CGX_3X_4 \quad 3'$$

wherein C is unmethylated, and X_1, X_2, X_3 and X_4 are nucleotides.

For economic reasons, preferably the immunostimulatory CpG DNA is in the range of between 8 to 40 base pairs in size if it is synthesized as an oligonucleotide. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids or as genomic DNA, e.g., DNA from bacteria, yeast, etc., which after being administered to a subject are degraded into oligonucleotides. Preferred immunostimulatory nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency by stimulating an antibody response in a subject) have a relatively high stimulation index with regard to B cell, monocyte and/or natural killer cell responses (e.g. cytokine, proliferative, lytic or other responses).

The stimulation index of a particular immunostimulatory CpG DNA can be tested in various immune cell assays. Preferably, the stimulation index of the immunostimulatory CpG DNA with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^{3}H uridine in a B cell culture, which has been contacted with a 20μM of ODN for 20h at 37°C and has been pulsed with 1μCi of ^{3}H uridine; and harvested and counted 4h later as described in detail in Example 1. For use *in vivo,* for example to treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, the immunostimulatory CpG DNA may be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

Immunostimulatory CpG nucleic acids may effect at least about 500 pg/ml of TNF-α, 15 pg/ml, IFN-γ, 70 pg/ml of GM-CSF, 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other immunostimulatory CpG DNAs may effect at least about 10 %, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or at least about 30, more preferably at least about 35 and most preferably at least about 40% 2C11 cell specific lysis as determined by the assay described in detail in Example 4.

A "nucleic acid" or "DNA" shall mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the term refers to ribonucleotides as well as oligodeoxyribonucleotides. The term shall also include polynucleosides (i.e. a polynucleotide minus the phosphate) and any other organic base containing polymer.

The term nucleic acid also encompasses nucleic acids or oligonucleotides with a covalently modified base and/or sugar. For example, they include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars not naturally found in nucleic acids such as arabinose instead of ribose. Thus the nucleic acids may be heterogeneous in backbone composition thereby containing any possible combination of polymer units linked together

such as peptide- nucleic acids (which have amino acid backbone with nucleic acid bases). In some embodiments the nucleic acids are homogeneous in backbone composition.

The substituted purines and pyrimidines of the nucleic acids include standard purines and pyrimidines such as cytosine as well as base analogs such as C-5 propyne substituted bases. Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymine, uracil 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties.

The nucleic acid is a linked polymer of bases or nucleotides. As used herein with respect to linked units of a nucleic acid, "linked" or "linkage" means two entities are bound to one another by any physicochemical means. Any linkage known to those of ordinary skill in the art, covalent or non-covalent, is embraced. Such linkages are well known to those of ordinary skill in the art. Natural linkages, which are those ordinarily found in nature connecting the individual units of a nucleic acid, are most common. The individual units of a nucleic acid may be linked, however, by synthetic or modified linkages.

Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), which are also referred to as isolated, or may be synthetic (e.g. produced by oligonucleotide synthesis). An isolated molecule is a molecule that is substantially pure and is free of other substances with which it is ordinarily found in nature or *in vivo* systems to an extent practical and appropriate for its intended use. In particular, the molecular species are sufficiently pure and are sufficiently free from other biological constituents of host cells so as to be useful in, for example, producing pharmaceutical preparations or sequencing if the molecular species is a nucleic acid, peptide, or polysaccharide. Because an isolated molecular species of the invention may be admixed with a pharmaceutically-acceptable carrier in a pharmaceutical preparation, the molecular species may comprise only a small percentage by weight of the preparation. The molecular species is nonetheless substantially pure in that it has been substantially separated from the substances with which it may be associated in living systems.

A "nucleic acid delivery complex" shall mean a nucleic acid molecule associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. B-cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g. cholesterol), a

lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes must be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form. A nucleic acid delivery complex is one form of delivery device. Others are described below.

"Palindromic sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEE'D'C'B'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double stranded structures. In some embodiments the oligonucleotide or immunostimulatory nucleic acid is a palindrome, in whole or in part. For instance, it may be a palindrome of 6 nucleotides or greater. In other embodiments the oligonucleotide or immunostimulatory nucleic acid does not include a palindrome of 6 nucleotides or greater.

A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter immunostimulatory nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic acid molecule has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the nucleic acid molecule becomes stabilized and therefore exhibits more activity.

Stabilized nucleic acid molecules of the instant invention may have a modified backbone. For use in immune stimulation, stabilized nucleic acid molecules may be phosphorothioate modified nucleic acid molecules (i.e. at least one of the phosphate oxygen's of the nucleic acid molecule is replaced by sulfur). Preferably the phosphate modification occurs at or near the 5' and/or 3' end of the nucleic acid molecule. A nucleic acid having a modified backbone is said to have at least one modified internucleotide linkage.

Thus, a preferred composition of the invention is a composition including an oligonucleotide having a phosphate modification at the 3' and 5' ends of the molecule with a phosphodiester central region. This preferred molecule is exemplified by the following formula:

$$5'\ Y_1N_1CGN_2Y_2\ 3'$$

wherein Y_1 and Y_2 are, independent of one another, nucleic acid molecules having between 1 and 10 nucleotides, and wherein Y_1 includes at least one modified internucleotide linkage and Y_2 includes at least one modified internucleotide linkage and wherein N_1 and N_2

are nucleic acid molecules, each independent of one another having between 0 and 20 nucleotides and in some embodiments, between 3 and 8 nucleotides, but wherein N_1CGN_2 has at least 6 nucleotides in total and wherein the nucleotides of N_1CGN_2 have a phosphodiester backbone. As described in more detail below, with respect to the experimental results, oligonucleotides having one or more phosphorothioate modified internucleotide linkages with a central region having one or more phosphodiester internucleotide linkages demonstrated unexpectedly high immune stimulating properties. The activity of these oligonucleotides was particularly high when the first two and last five internucleotide linkages include phosphate modifications and/or the oligonucleotide included Poly-G ends.

Y_1 and Y_2 are considered independent of one another. This means that each of Y_1 and Y_2 may or may not have different sequences and different backbone linkages from one another in the same molecule. The sequences vary, but in some cases Y_1 and Y_2 have a poly-G sequence. A poly-G sequence refers to at least 3 Gs in a row. In other embodiments the poly-G sequence refers to at least 4, 5, 6, 7, or 8 Gs in a row. In other embodiments Y_1 and Y_2 may be TCGTCG, TCGTCGT, or TCGTCGTT.

In some embodiments Y_1 and Y_2 have between 3 and 8 or between 4 and 7 nucleotides. At least one of these nucleotides includes a modified internucleotide linkage. In some embodiments Y_1 and Y_2 include at least two modified internucleotide linkages, and in other embodiments Y_1 and Y_2 include between two and five modified internucleotide linkages. In yet other embodiments Y_1 has two modified internucleotide linkages and Y_2 has five modified internucleotide linkages. As described below, oligonucleotides having this preferred structure have been found to have unexpectedly high immune stimulating activity. In other embodiments Y_1 has five modified internucleotide linkages and Y_2 has two modified internucleotide linkages.

In addition to stabilizing nucleic acid molecules, as reported further herein, phosphorothioate-modified nucleic acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated CpG dinucleotide as shown herein. As reported herein, unmethylated CpG containing nucleic acid molecules having a fully or nearly fully phosphorothioate-modified backbone have been found to preferentially activate B-cell activity, while unmethylated CpG containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages and monocytes), dendritic (DC), and NK

cells. Phosphorothioate CpG oligonucleotides with preferred human motifs are also strong activators of monocytic and NK cells. The preferred chimeric oligonucleotides of the invention primarily activate, NK cells, dendritic cells and monocytic cells.

Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acid molecules which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

A "subject" shall mean a human or vertebrate animal including a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rabbit, rat, mouse, etc.

Infectious disease, cancer and allergies are prevalent among human and non-human animals alike. For instance, cancer is one of the leading causes of death in companion animals (i.e., cats and dogs). Cancer usually strikes older animals which, in the case of house pets, have become integrated into the family. Forty-five % of dogs older than 10 years of age, are likely to succumb to the disease. The most common treatment options include surgery, chemotherapy and radiation therapy. Others treatment modalities which have been used with some success are laser therapy, cryotherapy, hyperthermia and immunotherapy. The choice of treatment depends on type of cancer and degree of dissemination. Unless the malignant growth is confined to a discrete area in the body, it is difficult to remove only malignant tissue without also affecting normal cells.

Malignant disorders commonly diagnosed in dogs and cats include but are not limited to lymphosarcoma, osteosarcoma, mammary tumors, mastocytoma, brain tumor, melanoma, adenosquamous carcinoma, carcinoid lung tumor, bronchial gland tumor, bronchiolar adenocarcinoma, fibroma, myxochondroma, pulmonary sarcoma, neurosarcoma, osteoma, papilloma, retinoblastoma, Ewing's sarcoma, Wilm's tumor, Burkitt's lymphoma, microglioma, neuroblastoma, osteoclastoma, oral neoplasia, fibrosarcoma, osteosarcoma and rhabdomyosarcoma. Other neoplasias in dogs include genital squamous cell carcinoma, transmissable veneral tumor, testicular tumor, seminoma, Sertoli cell tumor, hemangiopericytoma, histiocytoma, chloroma (granulocytic sarcoma), corneal papilloma, corneal squamous cell carcinoma, hemangiosarcoma, pleural mesothelioma, basal cell tumor, thymoma, stomach tumor, adrenal gland carcinoma, oral papillomatosis, hemangioendothelioma and cystadenoma. Additional malignancies diagnosed in cats include follicular lymphoma, intestinal lymphosarcoma, fibrosarcoma and pulmonary squamous cell carcinoma. The ferret, an ever-more popular house pet is known to develop insulinoma, lymphoma, sarcoma, neuroma, pancreatic islet cell tumor, gastric MALT lymphoma and gastric adenocarcinoma.

Neoplasias affecting agricultural livestock include leukemia, hemangiopericytoma and bovine ocular neoplasia (in cattle); preputial fibrosarcoma, ulcerative squamous cell carcinoma, preputial carcinoma, connective tissue neoplasia and mastocytoma (in horses); hepatocellular carcinoma (in swine); lymphoma and pulmonary adenomatosis (in sheep); pulmonary sarcoma, lymphoma, Rous sarcoma, reticulendotheliosis, fibrosarcoma, nephroblastoma, B-cell lymphoma and lymphoid leukosis (in avian species); retinoblastoma, hepatic neoplasia, lymphosarcoma (lymphoblastic lymphoma), plasmacytoid leukemia and swimbladder sarcoma (in fish), caseous lumphadenitis (CLA): chronic, infectious, contagious disease of sheep and goats caused by the bacterium Corynebacterium pseudotuberculosis, and contagious lung tumor of sheep caused by jaagsiekte.

In addition to the use of the CpG oligonucleotides to induce an antigen specific immune response in humans, the methods of the preferred embodiments are particularly well suited for treatment of birds such as hens, chickens, turkeys, ducks, geese, quail, and pheasant. Birds are prime targets for many types of infections. Hatching birds are exposed to pathogenic microorganisms shortly after birth. Although these birds are initially protected against pathogens by maternal derived antibodies, this protection is only temporary, and the bird's own immature immune system must begin to protect the bird against the pathogens. It is often desirable to prevent infection in young birds when they are most susceptible. It is also desirable to prevent against infection in older birds, especially when the birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the CpG oligonucleotide of the invention to birds to enhance an antigen-specific immune response when antigen is present.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yuasa et al., 1979, Avian Dis. 23:366-385). Since that time, CIAV has been detected in commercial poultry in all major poultry producing countries (van Bulow et al., 1991, pp.690-699 in Diseases of Poultry, 9th edition, Iowa State University Press).

CIAV infection results in a clinical disease, characterized by anemia, hemorrhage and immunosuppression, in young susceptible chickens. Atrophy of the thymus and of the bone marrow and consistent lesions of CIAV-infected chickens are also characteristic of CIAV infection. Lymphocyte depletion in the thymus, and occasionally in the bursa of Fabricius, results in immunosuppression and increased susceptibility to secondary viral, bacterial, or fungal infections which then complicate the course of the disease. The immunosuppression may cause aggravated disease after infection with one or more of Marek's disease virus (MDV), infectious bursal disease virus, reticuloendotheliosis virus, adenovirus, or reovirus. It has been reported that pathogenesis of MDV is enhanced by CIAV (DeBoer et al., 1989, p. 28 In Proceedings of the 38th Western Poultry Diseases Conference,

Tempe, Ariz.). Further, it has been reported that CIAV aggravates the signs of infectious bursal disease (Rosenberger et al., 1989, Avian Dis. 33:707-713). Chickens develop an age resistance to experimentally induced disease due to CAA. This is essentially complete by the age of 2 weeks, but older birds are still susceptible to infection (Yuasa, N. et al., 1979 supra; Yuasa, N. et al., Arian Diseases 24, 202-209, 1980). However, if chickens are dually infected with CAA and an immunosuppressive agent (IBDV, MDV etc.) age resistance against the disease is delayed (Yuasa, N. et al., 1979 and 1980 supra; Bulow von V. et al., J. Veterinary Medicine 33, 93-116, 1986). Characteristics of CIAV that may potentiate disease transmission include high resistance to environmental inactivation and some common disinfectants. The economic impact of CIAV infection on the poultry industry is clear from the fact that 10% to 30% of infected birds in disease outbreaks die.

Vaccination of birds, like other vertebrate animals can be performed at any age. Normally, vaccinations are performed at up to 12 weeks of age for a live microorganism and between 14-18 weeks for an inactivated microorganism or other type of vaccine. For in ovo vaccination, vaccination can be performed in the last quarter of embryo development. The vaccine may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, or by other mucosal delivery methods described herein. Thus, the CpG oligonucleotide of the invention can be administered to birds and other non-human vertebrates using routine vaccination schedules.

Cattle and livestock are also susceptible to infection. Disease which affect these animals can produce severe economic losses, especially amongst cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats.

Cows can be infected by bovine viruses. Bovine viral diarrhea virus (BVDV) is a small enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although, Pestiviruses were previously classified in the Togaviridae family, some studies have suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups (Francki, et al., 1991).

BVDV, which is an important pathogen of cattle can be distinguished, based on cell culture analysis, into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes. The NCP biotype is more widespread although both biotypes can be found in cattle. If a pregnant cow becomes infected with an NCP strain, the cow can give birth to a persistently infected and specifically immunotolerant calf that will spread virus during its lifetime. The persistently infected cattle can succumb to mucosal disease and both biotypes can then be isolated from the animal. Clinical manifestations can include abortion, teratogenesis, and respiratory problems, mucosal disease and mild diarrhea. In addition, severe thrombocytopenia,

associated with herd epidemics, that may result in the death of the animal has been described and strains associated with this disease seem more virulent than the classical BVDVs.

Equine herpesviruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal disease. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and central nervous system disorders. Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts for 8 to 10 days. Immunologically experienced mares may be reinfected via the respiratory tract without disease becoming apparent, so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of either sex at any age, leading to incoordination, weakness and posterior paralysis (Telford, E. A. R. et al., Virology 189, 304-316, 1992). Other EHV's include EHV-2, or equine cytomegalovirus, EHV-3, equine coital exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

Sheep and goats can be infected by a variety of dangerous microorganisms including visna-maedi.

Primates such as monkeys, apes and macaques can be infected by simian immunodeficiency virus. Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 36:1538-1541; Desrosiers et al. PNAS USA (1989) 86:6353-6357; Murphey-Corb et al. (1989) Science 246:1293-1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

Cats, both domestic and wild, are susceptible to infection with a variety of microorganisms. For instance, feline infectious peritonitis is a disease which occurs in both domestic and wild cats, such as lions, leopards, cheetahs, and jaguars. When it is desirable to prevent infection with this and other types of pathogenic organisms in cats, the methods of the invention can be used to vaccinate cats to protect them against infection.

Domestic cats may become infected with several retroviruses, including but not limited to feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncornavirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immune mediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

The discovery of feline T-lymphotropic lentivirus (also referred to as feline immunodeficiency) was first reported in Pedersen et al. (1987) Science 235:790-793. Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258; and Ackley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. USA 86:2448-2452 and 86:4355-4360.

Feline infectious peritonitis (FIP) is a sporadic disease occurring unpredictably in domestic and wild Felidae. While FIP is primarily a disease of domestic cats, it has been diagnosed in lions, mountain lions, leopards, cheetahs, and the jaguar. Smaller wild cats that have been afflicted with FIP include the lynx and caracal, sand cat, and pallas cat. In domestic cats, the disease occurs predominantly in young animals, although cats of all ages are susceptible. A peak incidence occurs between 6 and 12 months of age. A decline in incidence is noted from 5 to 13 years of age, followed by an increased incidence in cats 14 to 15 years old.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and expression of nucleic acids to which they are linked (e.g., an episome). Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Certain Unmethylated CpG Containing Nucleic Acids Have B Cell Stimulatory Activity As Shown in vitro and in vivo

In the course of investigating the lymphocyte stimulatory effects of two antisense oligonucleotides specific for endogenous retroviral sequences, using protocols described in the attached Examples 1 and 2, it was surprisingly found that two out of twenty-four "controls" (including various scrambled, sense, and mismatch controls for a panel of "antisense" ODN) also mediated B cell activation and IgM secretion, while the other "controls" had no effect.

Two observations suggested that the mechanism of this B cell activation by the "control" ODN may not involve antisense effects 1) comparison of vertebrate DNA sequences listed in GenBank showed no greater homology than that seen with non-

stimulatory ODN and 2) the two controls showed no hybridization to Northern blots with 10 μg of spleen poly A+ RNA. Resynthesis of these ODN on a different synthesizer or extensive purification by polyacrylamide gel electrophoresis or high pressure liquid chromatography gave identical stimulation, eliminating the possibility of an impurity. Similar stimulation was seen using B cells from C3H/HeJ mice, eliminating the possibility that lipopolysaccharide (LPS) contamination could account for the results.

The fact that two "control" ODN caused B cell activation similar to that of the two "antisense" ODN raised the possibility that all four ODN were stimulating B cells through some non-antisense mechanism involving a sequence motif that was absent in all of the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained CpG dinucleotides that were in a different sequence context from the nonstimulatory control.

To determine whether the CpG motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no CpG dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two originally synthesized as "antisense" (ODN 3D and 3M; Krieg, A.M. *J. Immunol.* *143*:2448 (1989)), were then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained CpG dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more CpG dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result from an antisense mechanism or impurity. ODN caused no detectable proliferation of γδ or other T cell populations.

Mitogenic ODN sequences uniformly became nonstimulatory if the CpG dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the CpG dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b,2b,3Dd, and 3Mb). Partial methylation of CpG motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a CpG motif is the essential element present in ODN that activate B cells.

In the course of these studies, it became clear that the bases flanking the CpG dinucleotide played an important role in determining the murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a CpG flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the CpG motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations that disturbed the motif reduced stimulation (e.g. Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d).

On the other hand, mutations outside the CpG motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to 1d; 3D to 3Dg; 3M to 3Me). For activation of human cells, the best flanking bases are slightly different (See Table 5).

Of those tested, ODNs shorter than 8 bases were non-stimulatory (e.g. Table 1, ODN 4e). Among the forty-eight 8 base ODN tested, the most stimulatory sequence identified was TCAACGTT (ODN 4) which contains the self complementary "palindrome" AACGTT. In further optimizing this motif, it was found that ODN containing Gs at both ends showed increased stimulation, particularly if the ODN were rendered nuclease resistant by phosphorothioate modification of the terminal internucleotide linkages. ODN 1585 (5' GGGGTCAACGTTGAGGGGGG 3' (SEQ ID NO:12)), in which the first two and last five internucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation induced by ODN 1638, which has the same sequence as ODN 1585 except that the 10 Gs at the two ends are replaced by 10 As. The effect of the G-rich ends is *cis*; addition of an ODN with poly G ends but no CpG motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated CpG were found to be more immunostimulatory.

Other octamer ODN containing a 6 base palindrome with a TpC dinucleotide at the 5' end were also active (e.g. Table 1, ODN 4b,4c). Other dinucleotides at the 5' end gave reduced stimulation (e.g. ODN 4f; all sixteen possible dinucleotides were tested). The presence of a 3' dinucleotide was insufficient to compensate for the lack of a 5' dinucleotide (e.g. Table 1, ODN 4g). Disruption of the palindrome eliminated stimulation in octamer ODN (e.g. Table 1, ODN 4h), but palindromes were not required in longer ODN.

Table 1: Oligonucleotide Stimulation of Mouse B Cells

ODN	Sequence (5' to 3')†	Stimulation Index' ^{3}H Uridine	Stimulation Index' IgM Production
1 (SEQ ID NO:13)	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a (SEQ. ID NO:4)	......T.....T.	1.2 ± 0.2	1.7 ± 0.5
1b (SEQ ID NO:14)	......Z.....T.	1.2 ± 0.1	1.8 ± 0.0
1c (SEQ ID NO:15)	.....T.....Z..	10.3 ± 4.4	9.5 ± 1.8
1d (SEQ ID NO:16)	..AT.....GAGC.	13.0 ± 2.3	18.3 ± 7.5
2 (SEQ ID NO:17)	ATGGAAGGTCCAGCGTTCTC	2.9 ± 0.2	13.6 ± 2.0
2a (SEQ ID NO:18)	..C..CTC..G..T.....	7.7 ± 0.8	24.2 ± 3.2
2b (SEQ ID NO:19)	..Z..CTC.ZG..Z.....	1.6 ± 0.5	2.8 ± 2.2
2c (SEQ ID NO:20)	..Z..CTC..G..T.....	3.1 ± 0.6	7.3 ± 1.4
2d (SEQ ID NO:21)	..C..CTC..G.....Z..	7.4 ± 1.4	27.7 ± 5.4

	2e (SEQ ID NO:22)	..........A.....	5.6 ± 2.0	ND
	3D (SEQ ID NO:23)	GAGAACGCTGGACCTTCCAT	4.9 ± 0.5	19.9 ± 3.6
	3Da(SEQ ID NO:24)	.....C.....	6.6 ± 1.5	33.9 ± 6.8
5	3Db(SEQ ID NO:25)	.....C.....G..	10.1 ± 2.8	25.4 ± 0.8
	3Dc(SEQ ID NO:26)	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
	3Dd(SEQ ID NO:27)	.....Z.....	1.2 ± 0.2	1.0 ± 0.4
	3De(SEQ ID NO:28)	.....Z.....	4.4 ± 1.2	18.8 ± 4.4
	3Df(SEQ ID NO:29)	.....A.....	1.6 ± 0.1	7.7 ± 0.4
10	3Dg(SEQ ID NO:30)	.....CC.G.ACTG.	6.1 ± 1.5	18.6 ± 1.5
	3M (SEQ ID NO:31)	TCCATGTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
	3Ma(SEQ ID NO:32)	.....CT.....	0.9 ± 0.1	1.8 ± 0.5
	3Mb(SEQ ID NO:33)	.....Z.....	1.3 ± 0.3	1.5 ± 0.6
15	3Mc(SEQ ID NO:34)	.....Z.....	5.4 ± 1.5	8.5 ± 2.6
	3Md(SEQ ID NO:35)	.....A..T.....	17.2 ± 9.4	ND
	3Me(SEQ ID NO:36)	.....C..A.	3.6 ± 0.2	14.2 ± 5.2
	4 (SEQ ID NO:56)	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
20	4a(SEQ ID NO:57)	....GC..	1.1 ± 0.2	1.5 ± 1.1
	4b(SEQ ID NO:58)	...GCGC.	4.5.± 0.2	9.6 ± 3.4
	4c(SEQ ID NO:59)	...TCGA.	2.7.± 1.0	ND
	4d(SEQ ID NO:60)	..TT..AA	1.3 ± 0.2	ND
	4e(SEQ ID NO:61)	-......	1.3 ± 0.2	1.1 ± 0.5
25	4f(SEQ ID NO:62)	C.....	3.9 ± 1.4	ND
	4g(SEQ ID NO:63)	--.....CT	1.4 ± 0.3	ND
	4h(SEQ ID NO:64)	C	1.2 ± 0.2	ND
	LPS		7.8 ± 2.5	4.8 ± 1.0

30

Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.
ND = not done.
CpG dinucleotides are underlined.
35 Dots indicate identity; dashes indicate deletions.
Z indicates 5 methyl cytosine.

Table 2. Identification of the optimal CpG motif for Murine IL-6 production and B cell activation.

40

ODN	SEQUENCE (5'-3')		IL-6 (pg/ml)[a]		SI[b]	IgM (ng/ml)[c]
			CH12.LX	SPLENIC B CELL		
512	(SEQ ID NO:37)	TCCATGTCGGTCCTGATGCT	1300 + 106	627 + 43	5.8 + 0.3	7315 + 1324
1637	(SEQ ID NO:38)	.....C.....	136 + 27	46 + 6	1.7 + 0.2	770 + 72
1615	(SEQ ID NO:39)	.....G.....	1201 + 155	850 + 202	3.7 + 0.3	3212 + 617
1614	(SEQ ID NO:40	.....A.....	1533 + 321	1812 + 103	10.8 + 0.6	7558 + 414
1636	(SEQ ID NO:41)	.....A.....	1181 + 76	947 + 132	5.4 + 0.4	3983 + 485
1634	(SEQ ID NO:42)	.....C.....	1049 + 223	1671 + 175	9.2 + 0.9	6256 + 261

1619	(SEQ ID NO:43)	.....T.....	1555 + 304	2908 + 129	12.5 + 1.0	8243 + 698
1618	(SEQ ID NO:44)	.....A..T.....	2109 + 291	2596 + 166	12.9 + 0.7	10425 + 674
1639	(SEQ ID NO:45)	.....AA..T.....	1827 + 83	2012 + 132	11.5 + 0.4	9489 + 103
1707	(SEQ ID NO:46)	.....A..TC.....	ND	1147 + 175	4.0 + 0.2	3534 + 217
1708	(SEQ ID NO:47)	.....CA..TG.....	ND	59 + 3	1.5 + 0.1	466 + 109

Dots indicate identity; CpG dinucleotides are underlined; ND= not done

[a]The experiment was done at least three times with similar results. The level of IL-6 of unstimulated control cultures of both CH12.LX and splenic B cells was $\leq$ 10 pg/ml. The IgM level of unstimulated culture was 547 $\pm$ 82 ng/ml. CpG dinucleotides are underlined and dots indicate identity.

[b][^{3}H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 $\pm$ 213.68 cpm). Cells were stimulated with 20 μM of various CpG O-ODN. Data present the mean $\pm$ SD of triplicates

[c]Measured by ELISA .

The kinetics of lymphocyte activation were investigated using mouse spleen cells. When the cells were pulsed at the same time as ODN addition and harvested just four hours later, there was already a two-fold increase in ^{3}H uridine incorporation. Stimulation peaked at 12-48 hours and then decreased. After 24 hours, no intact ODN were detected, perhaps accounting for the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with CpG ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude of stimulation was concentration dependent and consistently exceeded that of LPS under optimal conditions for both. Oligonucleotides containing a nuclease resistant phosphorothioate backbone were approximately two hundred times more potent than unmodified oligonucleotides.

Cell cycle analysis was used to determine the proportion of B cells activated by CpG-ODN. CpG-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone) and CD23+ (follicular) subpopulations were equally responsive to ODN- induced stimulation, as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that CpG-ODN induce essentially all B cells to enter the cell cycle.

Immunostimulatory Nucleic Acid Molecules Block Murine B Cell Apoptosis

Certain B cell lines such as WEHI-231 are induced to undergo growth arrest and/or apoptosis in response to crosslinking of their antigen receptor by anti-IgM (Jakway, J.P. et al., "Growth regulation of the B lymphoma cell line WEHI-231 by anti-immunoglobulin, lipopolysaccharide and other bacterial products" *J. Immunol. 137:* 2225 (1986); Tsubata, T., J. Wu and T. Honjo: B-cell apoptosis induced by antigen receptor crosslinking is blocked by

a T-cell signal through CD40." *Nature 364*: 645 (1993)). WEHI-231 cells are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the CpG motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that CpG ODN induce Bcl-x and *myc* expression, which may account for the protection from apoptosis. Also, CpG nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by CpG DNA.

Induction of Murine Cytokine Secretion by CpG motifs in Bacterial DNA or Oligonucleotides.

As described in Example 9, the amount of IL-6 secreted by spleen cells after CpG DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for *in vitro* studies following preliminary studies showing that T cells contribute little or nothing to the IL-6 produced by CpG DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with *E. coli* DNA but not in cells cultured with calf thymus DNA. To confirm that the increased IL-6 production observed with *E. coli* DNA was not due to contamination by other bacterial products, the DNA was digested with DNAse prior to analysis. DNAse pretreatment abolished IL-6 production induced by *E. coli* DNA (Table 3). In addition, spleen cells from LPS-nonresponsive C3H/HeJ mouse produced similar levels of IL-6 in response to bacterial DNA. To analyze whether the IL-6 secretion induced by *E. coli* DNA was mediated by the unmethylated CpG dinucleotides in bacterial DNA, methylated *E. coli* DNA and a panel of synthetic ODN were examined. As shown in Table 3, CpG ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while CpG methylated *E. coli* DNA, or ODN containing methylated CpG (ODN 5f) or no CpG (ODN 5d) did not. Changes at sites other than CpG dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of CpG ODN. Methylation of a single CpG in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table 3).

Table 3. Induction of Murine IL-6 secretion by CpG motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	$\leq$10
calf thymus DNA + DNase	$\leq$10
E. coli DNA	1169.5 $\pm$ 94.1
E. coli DNA + DNase	$\leq$10

CpG methylated E. coli DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10

ODN 5a	SEQ. ID. No:1	ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0
5b	SEQ. ID. No:2	.....AGG....A.....	1124.5 ± 126.2
5c	SEQ. ID. No:3	..C.....G.....	1783.0 ± 189.5
5d	SEQ. ID. No:4	.... AGG..C..T.....	≤10
5e	SEQ. ID. No:5	..C.....G..Z.....	851.1 ± 114.4
5f	SEQ. ID. No:6	..Z.....ZG..Z.....	≤10
5g	SEQ. ID. No:7	..C.....G.....Z..	1862.3 + 87.26

T cell depleted spleen cells from DBA/2 mice were stimulated with phosphodiester modified oligonucleotides (O-ODN) (20 μM), calf thymus DNA (50 μg/ml) or *E. coli* DNA (50 μg/ml) with or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. CpG dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

Identification of the optimal CpG motif for induction of Murine IL-6 and IgM secretion and B cell proliferation.

To evaluate whether the optimal B cell stimulatory CpG motif was identical with the optimal CpG motif for IL-6 secretion, a panel of ODN in which the bases flanking the CpG dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and IL-6 secretion, using both splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif is composed of an unmethylated CpG flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purine to pyrimidine or 3' pyrimidine to purine significantly reduced its effects. Changes in 5' purines to C were especially deleterious, but changes in 5' purines to T or 3' pyrimidines to purines had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal CpG motif for induction of IL-6 secretion is TGACGTT, which is identical with the optimal mitogenic and IgM-inducing CpG motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

Titration of induction of Murine IL-6 Secretion by CpG motifs.

Bacterial DNA and CpG ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent manner, but vertebrate DNA and non-CpG ODN did not (Fig. 1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of CpG O-ODN. The maximum levels of IL-6 induced by bacterial DNA and CpG ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater

than those seen after stimulation by LPS (0.35 ng/ml) (Fig. 1A). To evaluate whether CpG ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. CpG S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as CpG O-ODN while non-CpG S-ODN failed to induce IL-6 (Fig. 1C). CpG S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicated that the nuclease-resistant DNA backbone modification retains the sequence specific ability of CpG DNA to induce IL-6 secretion and that CpG S-ODN are more than 80-fold more potent than CpG O-ODN in this assay system.

Induction of Murine IL-6 secretion by CpG DNA in vivo.

To evaluate the ability of bacterial DNA and CpG S-ODN to induce IL-6 secretion *in vivo*, BALB/c mice were injected iv. with 100 μg of *E. coli* DNA, calf thymus DNA, or CpG or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the *E. coli* DNA injected group was approximately 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). CpG S-ODN also induced IL-6 secretion *in vivo*. The IL-6 level in the sera from CpG S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated group (Table 4).

Table 4. Secretion of Murine IL-6 induced by CpG DNA stimulation *in vivo*.

Stimulant	IL-6 (pg/ml)
PBS	< 50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	< 50
CpG S-ODN	20715 ± 606
non-CpG S-ODN	< 50

Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μg of *E. coli* DNA or calf thymus DNA, or 500 μg of CpG S-ODN or non-CpG control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the CpG S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. NO:48) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. NO:49). Note that although there is a CpG in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean ± SD of duplicates. The experiment was done at least twice with similar results.

Kinetics of Murine IL-6 secretion after stimulation by CpG motifs in vivo.

To evaluate the kinetics of induction of IL-6 secretion by CpG DNA *in vivo*, BALB/c mice were injected iv. with CpG or control non-CpG S-ODN. Serum IL-6 levels were

significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the CpG S-ODN injected group (Figure 2). IL-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to CpG DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S-ODN or PBS injected groups (Figure 2).

Tissue distribution and kinetics of IL-6 mRNA expression induced by CpG motifs in vivo.

As shown in Figure 2, the level of serum IL-6 increased rapidly after CpG DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression *in vivo* after CpG DNA stimulation, BALB/c mice were injected iv with CpG or non-CpG S-ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown in Figure 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of CpG S-ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after stimulation (Figure 3A). Splenic IL-6 mRNA peaked at 2 hr after stimulation and then gradually decreased (Figure 3A). Thymus IL-6 mRNA peaked at 1 hr post-injection and then gradually decreased (Figure 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after CpG S-ODN injection but then returned to basal level. In response to CpG S-ODN, liver , spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

Patterns of Murine Cytokine Expression Induced by CpG DNA

In vivo or in whole spleen cells, no significant increase in the protein levels of the following interleukins: IL-2, IL-3, IL-4, IL-5, or IL-10 was detected within the first six hours (Klinman, D.M. et al., (1996) *Proc. Natl. Acad. Sci. USA* 93:2879-2883). However, the level of TNF-α is increased within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with CpG ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two hours.

Table 5. Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'-3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
512 SEQ ID NO:37	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250

1637 SEQ ID NO:38	......C.....	550	16	7.8	15.6	35
1615 SEQ ID NO:39	.....G.....	600	145	7.8	45	250
1614 SEQ ID NO:40	......A.....	550	31	0	50	250
1636 SEQ ID NO:41	........A.....	325	250	35	40	0
1634 SEQ ID NO:42	........C.....	300	400	40	85	200
1619 SEQ ID NO:43	........T.....	275	450	200	80	>500
1618 SEQ ID NO:44	......A..T.....	300	60	15.6	15.6	62
1639 SEQ ID NO:45	.....AA..T.....	625	220	15.6	40	60
1707 SEQ ID NO:46	......A..TC.....	300	70	17	0	0
1708 SEQ ID NO:47	.....CA..TG.....	270	10	17	0	0

dots indicate identity; CpG dinucleotides are underlined

[1]measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum with the indicated oligodeoxynucleotides (12 μg/ml) for 4 hr in the case of TNF-α or 24 hr for the other cytokines before supernatant harvest and assay. Data are presented as the level of cytokine above that in wells with no added oligodeoxynucleotide.

CpG DNA induces cytokine secretion by human PBMC, specifically monocytes The same panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by CpG motifs to express cytokine (or proliferate), and to identify the CpG motif(s) responsible. Oligonucleotide 1619 (GTCGTT) was the best inducer of TNF-α and IFN-γ secretion, and was closely followed by a nearly identical motif in oligonucleotide 1634 (GTCGCT) (Table 5). The motifs in oligodeoxynucleotides 1637 and 1614 (GCCGGT and GACGGT) led to strong IL-6 secretion with relatively little induction of other cytokines. Thus, it appears that human lymphocytes, like murine

lymphocytes, secrete cytokines differentially in response to CpG dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain CpG oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC and CACGTG respectively).

The cells responding to the DNA appear to be monocytes, since the cytokine secretion is abolished by treatment of the cells with L-leucyl-L-leucine methyl ester (L-LME), which is selectively toxic to monocytes (but also to cytotoxic T lymphocytes and NK cells), and does not affect B cell Ig secretion (Table 6, and data not shown). The cells surviving L-LME treatment had >95% viability by trypan blue exclusion, indicating that the lack of a cytokine response among these cells did not simply reflect a nonspecific death of all cell types. Cytokine secretion in response to *E. coli* (EC) DNA requires unmethylated CpG motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination of the DNA cannot explain the results since the level of contamination was identical in the native and methylated DNA, and since addition of twice the highest amount of contaminating LPS had no effect (not shown).

Table 6. CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α (pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 μg/ml)	900	12,000	700	1560
EC DNA (5 μg/ml)	850	11,000	400	750
EC DNA (0.5 μg/ml)	500	ND	200	0
EC DNA (0.05 μg/ml)	62.5	10,000	15.6	0
EC DNA (50 μg/ml) + L-LME[2]	0	ND	ND	ND
EC DNA (10 μg/ml) Methyl.[3]	0	5	ND	ND
CT DNA (50 μg/ml)	0	600	0	0

[1]Levels of all cytokines were determined by ELISA using Quantikine kits from R&D Systems as described in the previous table. Results are representative using PBMC from different donors.

[2]Cells were pretreated for 15 min. with L-leucyl-L-leucine methyl ester (M-LME) to determine whether the cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells).

[3]EC DNA was methylated using 2U/μg DNA of CpG methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a negative control, samples were included containing twice the maximal amount of LPS contained in the highest concentration of EC DNA which failed to induce detectable cytokine production under these experimental conditions.

ND = not done

The loss of cytokine production in the PBMC treated with L-LME suggested that monocytes may be responsible for cytokine production in response to CpG DNA. To test this hypothesis more directly, the effects of CpG DNA on highly purified human monocytes and macrophages was tested. As hypothesized, CpG DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-CpG DNA did not (Table 7).

Table 7. CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α (pg/ml)
Cells alone	0	0	0
CT DNA (50 μg/ml)	0	0	0
EC DNA (50 μg/ml)	2000	15.6	1000

Biological Role of IL-6 in Inducing Murine IgM Production in Response to CpG Motifs.

The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post CpG stimulation, precedes IgM secretion. Since the optimal CpG motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the CpG motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited *in vitro* IgM production mediated by CpG ODN in a dose-dependent manner but a control antibody did not (Figure 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the CpG-induced B cell proliferation (Figure 4B).

Increased transcriptional activity of the IL-6 promoter in response to CpG DNA.

The increased level of IL-6 mRNA and protein after CpG DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was upregulated in B cells cultured with CpG

ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to CpG DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottratz, S.T. et al., 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. *J.Clin. Invest.* 93:944). CAT assays were performed after stimulation with various concentrations of CpG or non-CpG ODN. As shown in Figure 5, CpG ODN induced increased CAT activity in dose-dependent manner while non-CpG ODN failed to induce CAT activity. This confirms that CpG induces the transcriptional activity of the IL-6 promoter.

Dependence of B cell activation by CpG ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages.

To determine whether partial sulfur modification of the ODN backbone would be sufficient to enhance B cell activation, the effects of a series of ODN with the same sequence, but with differing numbers of S internucleotide linkages at the 5' and 3' ends were tested. Based on previous studies of nuclease degradation of ODN, it was determined that at least two phosphorothioate linkages at the 5' end of ODN were required to provide optimal protection of the ODN from degradation by intracellular exo- and endo- nucleases. Only chimeric ODN containing two 5' phosphorothioate-modified linkages, and a variable number of 3' modified linkages were therefore examined.

The lymphocyte stimulating effects of these ODN were tested at three concentrations (3.3, 10, and 30 μM) by measuring the total levels of RNA synthesis (by ^{3}H uridine incorporation) or DNA synthesis (by ^{3}H thymidine incorporation) in treated spleen cell cultures (Example 10). O-ODN (0/0 phosphorothioate modifications) bearing a CpG motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 μM (Example 10). However, when this sequence was modified with two S linkages at the 5' end and at least three S linkages at the 3' end, significant stimulation was seen at a dose of 3.3 μM. At this low dose, the level of stimulation showed a progressive increase as the number of 3' modified bases was increased, until this reached or exceeded six, at which point the stimulation index began to decline. In general, the optimal number of 3' S linkages for spleen cell stimulation was five. At all three concentrations tested in these experiments, the S-ODN was less stimulatory than the optimal chimeric compounds.

Dependence of CpG-mediated lymphocyte activation on the type of backbone modification.

Phosphorothioate modified ODN (S-ODN) are far more nuclease resistant than phosphodiester modified ODN (O-ODN). Thus, the increased immune stimulation caused by S-ODN and S-O-ODN (i.e. chimeric phosphorothioate ODN in which the central linkages

are phosphodiester, but the two 5' and five 3' linkages are phosphorothioate modified) compared to O-ODN may result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by CpG ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either methylphosphonate (MP-), methylphosphorothioate (MPS-), phosphorothioate (S-), or phosphorodithioate (S_2-) internucleotide linkages were tested (Example 10). These studies showed that despite their nuclease resistance, MP-O-ODN were actually less immune stimulatory than O-ODN. However, combining the MP and S modifications by replacing both nonbridging O molecules with 5' and 3' MPS internucleotide linkages restored immune stimulation to a slightly higher level than that triggered by O-ODN.

S-O-ODN were far more stimulatory than O-ODN, and were even more stimulatory than S-ODN, at least at concentrations above 3.3 μM. At concentrations below 3 μM, the S-ODN with the 3M sequence was more potent than the corresponding S-O-ODN, while the S-ODN with the 3D sequence was less potent than the corresponding S-O-ODN (Example 10). In comparing the stimulatory CpG motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the CpG is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the CpG in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it was found that the sequence requirement for immune stimulation is more stringent for S-ODN than for S-O- or O-ODN. S-ODN with poor matches to the optimal CpG motif cause little or no lymphocyte activation (e.g. Sequence 3D). However, S-ODN with good matches to the motif, most critically at the positions immediately flanking the CpG, are more potent than the corresponding S-O-ODN (e.g. Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater than 3 μM) the peak effect from the S-O-ODN is greater (Example 10).

S_2-O-ODN were remarkably stimulatory, and caused substantially greater lymphocyte activation than the corresponding S-ODN or S-O-ODN at every tested concentration.

The increased B cell stimulation seen with CpG ODN bearing S or S_2 substitutions could result from any or all of the following effects: nuclease resistance, increased cellular uptake, increased protein binding, and altered intracellular localization. However, nuclease resistance can not be the only explanation, since the MP-O-ODN were actually less stimulatory than the O-ODN with CpG motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao et al., (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate,

and mixed phosphorothioate and methylphosphonate oligonucleotides. (Antisense Research and Development 3, 53-66; Zhao et al., (1994) Stage specific oligonucleotide uptake in murine bone marrow B cell precursors. Blood 84, 3660-3666.) The highest cell membrane binding and uptake was seen with S-ODN, followed by S-O-ODN, O-ODN, and MP-ODN. This differential uptake correlates well with the degree of immune stimulation.

Unmethylated CpG Containing Oligos Have NK Cell Stimulatory Activity

Experiments were conducted to determine whether CpG containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with CpG ODN 1 and 3Dd was observed. In contrast, there was relatively no induction in effectors that had been treated with non-CpG control ODN.

Table 8. Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target		% 2C11 Specific Lysis Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	-1.1	-1.4	15.3	16.6
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-CpG ODN	-1.6	-1.7	14.8	15.4

Induction of NK activity by DNA containing CpG motifs, but not by non-CpG DNA.

Bacterial DNA cultured for 18 hrs. at 37°C and then assayed for killing of K562 (human) or Yac-1 (mouse) target cells induced NK lytic activity in both mouse spleen cells depleted of B cells and human PBMC, but vertebrate DNA did not (Table 9). To determine whether the stimulatory activity of bacterial DNA may be a consequence of its increased level of unmethylated CpG dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no CpG dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated CpG dinucleotide. No difference was observed in the stimulatory effects of ODN in which the CpG was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the CpG was flanked by two 5' purines or a 5' GpT dinucleotide and

two 3' pyrimidines. Kinetic experiments demonstrated that NK activity peaked around 18 hrs. after addition of the ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by CpG DNA, leading to the production of IL-12, TNF-α, and IFN-α/β (Example 11).

Table 9

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

DNA or Cytokine Added			LU/10^6 Mouse Cells	Human Cells
Expt. 1	None		0.00	0.00
	IL-2		16.68	15.82
	E.Coli. DNA		7.23	5.05
	Calf thymus DNA		0.00	0.00
Expt. 2	None		0.00	3.28
	1585 ggGGTCAACGTTGAGGgggg	(SEQ ID NO:12)	7.38	17.98
	1629 ----------gtc----------	(SEQ ID NO:50)	0.00	4.4
Expt. 3	None		0.00	
	1613 GCTAGACGTTAGTGT	(SEQ ID NO:51)	5.22	
	1769 ----------Z----------	(SEQ ID NO:52)	0.02	ND
	1619 TCCATGTCGTTCCTGATGCT	(SEQ ID NO:15)	3.35	
	1765 ----------Z----------	(SEQ ID NO:53)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide linkages which, in titration experiments, were more than 20 times as potent as non-modified ODN, depending on the flanking bases. Poly G ends (g) were used in some ODN, because they significantly increase the level of ODN uptake.

From all of these studies, a more complete understanding of the immune effects of CpG DNA has been developed, which is summarized in Figure 6.

Identification of B cell and monocyte/NK cell-specific oligonucleotides

As shown in Figure 6, CpG DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which CpG DNA

activates these cell types. For example, both require NFκB activation as explained further below.

In further studies of different immune effects of CpG DNA, it was found that there is more than one type of CpG motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and natural killer (NK) cell activation, while oligo 1758 is a weak B cell activator, but still induces excellent NK responses (Table 10).

Table 10. Different CpG motifs stimulate optimal murine B cell and NK activation

ODN	Sequence	B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT (SEQ.ID.NO:54)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT (SEQ.ID.NO.55)	1,747	6.66
NONE		367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.

[1]Measured by ^{3}H thymidine incorporation after 48 hr culture with oligodeoxynucleotides at a 200 nM concentration as described in Example 1.

[2]Measured in lytic units.

Teleological Basis of Immunostimulatory, Nucleic Acids

Vertebrate DNA is highly methylated and CpG dinucleotides are underrepresented. However, the stimulatory CpG motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported to induce B cell proliferation and immunoglobulin (Ig) production, while mammalian DNA does not (Messina, J.P. et al., *J. Immunol. 147*:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with CpG methylase was found to abolish mitogenicity, demonstrates that the difference in CpG status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion; that unmethylated CpG dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

Teleologically, it appears likely that lymphocyte activation by the CpG motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be present in many anatomic regions and areas of inflammation due to apoptosis (cell death), would generally induce little or no lymphocyte activation due to CpG suppression and methylation. However, the presence of bacterial DNA containing unmethylated CpG motifs can cause lymphocyte activation precisely in infected

anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the CpG pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would receive one activation signal through cell membrane Ig and a second signal from bacterial DNA, and would therefore tend to be preferentially activated. The interrelationship of this pathway with other pathways of B cell activation provide a physiologic mechanism employing a polyclonal antigen to induce antigen-specific responses.

However, it is likely that B cell activation would not be totally nonspecific. B cells bearing antigen receptors specific for bacterial products could receive one activation signal through cell membrane Ig, and a second from bacterial DNA, thereby more vigorously triggering antigen specific immune responses. As with other immune defense mechanisms, the response to bacterial DNA could have undesirable consequences in some settings. For example, autoimmune responses to self antigens would also tend to be preferentially triggered by bacterial infections, since autoantigens could also provide a second activation signal to autoreactive B cells triggered by bacterial DNA. Indeed the induction of autoimmunity by bacterial infections is a common clinical observance. For example, the autoimmune disease systemic lupus erythematosus, which is: i) characterized by the production of anti-DNA antibodies; ii) induced by drugs which inhibit DNA methyltransferase (Cornacchia, E.J. et al., *J. Clin. Invest. 92*:38 (1993)); and iii) associated with reduced DNA methylation (Richardson, B., L. et al., *Arth. Rheum 35*:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by CpG motifs, as well as by binding of bacterial DNA to antigen receptors.

Further, sepsis, which is characterized by high morbidity and mortality due to massive and nonspecific activation of the immune system may be initiated by bacterial DNA and other products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes. Further evidence of the role of CpG DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) *The Journal of Immunology* 156:4570-4575.

Proposed Mechanisms of Action

Unlike antigens that trigger B cells through their surface Ig receptor, CpG-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a CpG motif was performed as described in Zhao, Q et al.,(*Antisense Research and Development 3*:53-66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for CpG

ODN. Rather than acting through the cell membrane, that data suggests that unmethylated CpG containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. CpG ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

Recent data indicate the involvement of the transcription factor NFκB as a direct or indirect mediator of the CpG effect. For example, within 15 minutes of treating B cells or monocytes with CpG DNA, the level of NFkB binding activity is increased (Figure 7). However, it is not increased by DNA that does not contain CpG motifs. In addition, it was found that two different inhibitors of NFκB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by CpG DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFκB activation is required for both cell types.

There are several possible mechanisms through which NFκB can be activated. These include through activation of various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after CpG DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the CpG induced activation. However, CpG DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as detected by the sensitive fluorescent dye dihydrorhodamine 123 as described in Royall, J.A., and Ischiropoulos, H. (*Archives of Biochemistry and Biophysics* 302:348-355 (1993)). Moreover, inhibitors of the generation of these reactive oxygen species completely block the induction of NFκB and the later induction of cell proliferation and cytokine secretion by CpG DNA.

Working backwards, the next question was how CpG DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and plasmid or bacterial DNA are taken up by cells into endosomes. These endosomes rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which CpG DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and bafilomycin, which work through different mechanisms. Figure 8A shows the results from a flow cytometry study using mouse B cells with the dihydrorhodamine 123 dye to determine levels of reactive oxygen species. The dye only sample in Panel A of the figure shows the background level of cells positive for the dye at 28.6%. As expected, this level of reactive oxygen species was greatly increased to 80% in the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the CpG oligo also showed an increase in the level of reactive oxygen

species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the CpG was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

In the presence of chloroquine, the results are very different (Figure 8B). Chloroquine slightly lowers the background level of reactive oxygen species in the cells such that the untreated cells in Panel A have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with CpG DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and ionomycin (Panel E). This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with CpG DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the activation of NFκB by CpG DNA as well as the subsequent proliferation and induction of cytokine secretion.

Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing CpG motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind CpG DNA, we used electrophoretic mobility shift assays (EMSA) with 5' radioactively labeled oligonucleotides with or without CpG motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have CpG motifs, but not to oligonucleotides that lack CpG motifs or to oligonucleotides in which the CpG motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFκB binding site was added. This suggests that an NFκB or related protein is a component of a protein or protein complex that binds the stimulatory CpG oligonucleotides.

No activation of CREB/ATF proteins was found at time points where NFκB was strongly activated. These data therefore do not provide proof that NFκB proteins actually bind to the CpG nucleic acids, but rather that the proteins are required in some way for the CpG activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal CpG motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFκB activation.

Alternatively, it is very possible that the CpG nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate NFκB activation when CD40 is cross-linked. Examples of such TRAF proteins include TRAF-2 and TRAF-5.

Method for Making Immunostimulatory Nucleic Acids

For use in the instant invention, nucleic acids can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the β-cyanoethyl phosphoramidite method (S.L. Beaucage and M.H. Caruthers, (1981) *Tet. Let. 22*:1859); nucleoside H-phosphonate method (Garegg et al., (1986) *Tet. Let. 27*: 4051-4054; Froehler et al., (1986) *Nucl. Acid. Res. 14*: 5399-5407; Garegg et al., (1986) *Tet. Let. 27*: 4055-4058, Gaffney et al., (1988) *Tet. Let. 29*:2619-2622). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, oligonucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

For use *in vivo*, nucleic acids are preferably relatively resistant to degradation (e.g. via endo- and exo- nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl- phosphonates can be made e.g. as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A. (1990) *Chem. Rev.* 90:544; Goodchild, J. (1990) *Bioconjugate Chem.* 1:165). 2'-O-methyl nucleic acids with CpG motifs also cause immune activation, as do ethoxy-modified CpG nucleic acids. In fact, no backbone modifications have been found that completely abolish the CpG effect, although it is greatly reduced by replacing the C with a 5-methyl C.

For administration in vivo, nucleic acids may be associated with a molecule that results in higher affinity binding to target cell (e.g. B-cell, monocytic cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex". Nucleic acids can be ionically, or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or crosslinking agents can be used e.g. protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

Therapeutic Uses of Immunostimulatory Nucleic Acid Molecules

Based on their immunostimulatory properties, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be administered to a subject in vivo to prevent or treat an "immune system deficiency" such as a cancer, an infectious disease or an allergic or asthmatic disease. Alternatively, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells, dendritic cells, or NK cells) obtained from a subject *ex vivo* and activated lymphocytes can then be reimplanted in the subject.

As reported herein, in response to unmethylated CpG containing nucleic acid molecules, an increased number of immune cells secrete IL-6, IL-12, IFNγ, IFNα, IFNβ, IL-1, IL-3, IL-10, TNFα, TNFβ, GM-CSF, RANTES, and probably others. Additionally, increased IL-6 expression was found to occur in B cells, $CD4^+$ T cells and monocytic cells.

Immunostimulatory nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the immunostimulatory nucleic acid molecule is administered slightly before or after or at the same time as the vaccine. A conventional adjuvant may optionally be administered in conjunction with the vaccine, which is minimally comprised of an antigen, as the conventional adjuvant may further improve the vaccination by enhancing antigen absorption. A vaccine refers to an antigen, alone or in combination with an adjuvant (other than the immunostimulatory nucleic acid).

When the vaccine is a DNA vaccine at least two components determine its efficacy. First, the antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains CpG motifs, it functions as an adjuvant for the vaccine. Thus, CpG DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of CpG DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the costimulatory effects on B cells.

Thus, the immunostimulatory nucleic acid may be administered in conjunction with an antigen. An "antigen" as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide mimics of polysaccharides, lipids, glycolipids, carbohydrates, viruses and viral extracts and muticellular organisms such as parasites and allergens. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens (e.g., viral antigens, bacterial antigens, parasitic antigens, and fungal antigens), and allergens.

Immunostimulatory oligonucleotides and unmethylated CpG containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. alum), which are inert when injected alone and are thought to work through absorbing the antigen and thereby presenting it more effectively to immune cells. Further, conventional adjuvants only work for certain antigens, only induce a Th2 immune response, and are very poor at inducing cellular immune responses (Th1). For many pathogens, the Th2 response contributes little to protection, and can even be detrimental.

In addition, an immunostimulatory oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness of the malignant cells to subsequent chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. CpG nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). The chimeric oligonucleotides of the invention are particularly useful for treating cancer. Induction of NK activity and ADCC may be beneficial in cancer immunotherapy, alone or in conjunction with other treatments.

As used herein, a "cancer medicament" refers to a agent which is administered to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medicament is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, and cancer vaccines. The methods of the invention are intended to embrace the use of more than one cancer medicament along with the immunostimulatory nucleic acids in some embodiments. As an example, where appropriate, the immunostimulatory nucleic acids may be administered with a both a chemotherapeutic agent and an immunotherapeutic agent. Alternatively, the cancer medicament may embrace an immunotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine all administered to one subject for the purpose of treating a subject having a cancer or at risk of developing a cancer.

Cancer medicaments function in a variety of ways. Some cancer medicaments work by targeting physiological mechanisms that are specific to tumor cells. Examples include the targeting of specific genes and their gene products (i.e., proteins primarily) which are mutated in cancers. Such genes include but are not limited to oncogenes (e.g., Ras, Her2, bcl-2), tumor suppressor genes (e.g., EGF, p53, Rb), and cell cycle targets (e.g., CDK4, p21, telomerase). Cancer medicaments can alternately target signal transduction pathways and molecular mechanisms which are altered in cancer cells. Targeting of cancer cells via the epitopes expressed on their cell surface is accomplished through the use of monoclonal antibodies. This latter type of cancer medicament is generally referred to herein as immunotherapy.

Other cancer medicaments target cells other than cancer cells. For example, some medicaments prime the immune system to attack tumor cells (i.e., cancer vaccines). Still other medicaments, called angiogenesis inhibitors, function by attacking the blood supply of solid tumors. Since the most malignant cancers are able to metastasize (i.e., exist the primary tumor site and seed a distal tissue, thereby forming a secondary tumor), medicaments that impede this metastasis are also useful in the treatment of cancer. Angiogenic mediators include basic FGF, VEGF, angiopoietins, angiostatin, endostatin, TNFα, TNP-470, thrombospondin-1, platelet factor 4, CAI, and certain members of the integrin family of proteins. One category of this type of medicament is a metalloproteinase inhibitor, which inhibits the enzymes used by the cancer cells to exist the primary tumor site and extravasate into another tissue.

Some cancer cells are antigenic and thus can be targeted by the immune system. In one aspect, the combined administration of immunostimulatory nucleic acids and cancer medicaments, particularly those which are classified as cancer immunotherapies, is useful for stimulating a specific immune response against a cancer antigen. A "cancer antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens, such as those present in cancer vaccines or those used to prepare cancer immunotherapies, can be prepared from crude cancer cell extracts, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, or by partially purifying the antigens, using recombinant technology, or de novo synthesis of known antigens. Cancer antigens can be used in the form of immunogenic

portions of a particular antigen or in some instances a whole cell or a tumor mass can be used as the antigen. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

The use of immunostimulatory nucleic acids in conjunction with immunotherapeutic agents such as monoclonal antibodies is able to increase long-term survival through a number of mechanisms including significant enhancement of ADCC and activation of natural killer (NK) cells. The nucleic acids when used in combination with monoclonal antibodies serve to reduce the dose of the antibody required to achieve a biological result, and to greatly enhance the magnitude of the biological result.

Examples of cancer immunotherapies which are currently being used or which are in development are listed in the Table below.

Cancer Immunotherapies in Development or on the Market

MARKETER	BRAND NAME (GENERIC NAME)	INDICATION
IDEC/Genentech, Inc./Hoffmann-LaRoche (first monoclonal antibody licensed for the treatment of cancer in the U.S.)	Rituxan™ (rituximab, Mabthera) (IDEC-C2B8, chimeric murine/human anti-CD20 MAb)	non-Hodgkin's lymphoma
Genentech/Hoffmann-La Roche	Herceptin, anti-Her2 hMAb	Breast/ovarian
Cytogen Corp.	Quadramet (CYT-424) radiotherapeutic agent	Bone metastases
Centocor/Glaxo/Ajinomoto	Panorex® (17-1A) (murine monoclonal antibody)	Adjuvant therapy for colorectal (Dukes-C)
Centocor/Ajinomoto	Panorex® (17-1A) (chimeric murine monoclonal antibody)	Pancreatic, lung, breast, ovary
IDEC	IDEC-Y2B8 (murine, anti-CD20 MAb labeled with Yttrium-90)	non-Hodgkin's lymhoma
ImClone Systems	BEC2 (anti-idiotypic MAb, mimics the GD_3 epitope) (with BCG)	Small cell lung
ImClone Systems	C225 (chimeric monoclonal antibody to epidermal growth factor receptor (EGFr))	Renal cell
Techniclone International/Alpha Therapeutics	Oncolym (Lym-1 monoclonal antibody linked to 131 iodine)	non-Hodgkin's lymphoma
Protein Design Labs	SMART M195 Ab, humanized	Acute myleoid leukemia
Techniclone Corporation/Cambridge Antibody Technology	^{131}I LYM-1 (Oncolym™)	non-Hodgkin's lymphoma
Aronex Pharmaceuticals, Inc.	ATRAGEN®	Acute promyelocytic leukemia
ImClone Systems	C225 (chimeric anti-EGFr monoclonal antibody) + cisplatin or radiation	Head & neck, non-small cell lung cancer

Altarex, Canada	Ovarex (B43.13, anti-idiotypic CA125, mouse MAb)	Ovarian
Coulter Pharma (Clinical results have been positive, but the drug has been associated with significant bone marrow toxicity)	Bexxar (anti-CD20 Mab labeled with ^{131}I)	non-Hodgkin's lymphoma
Aronex Pharmaceuticals, Inc.	ATRAGEN®	Kaposi's sarcoma
IDEC Pharmaceuticals Corp./Genentech	Rituxan™ (MAb against CD20) pan-B Ab in combo. with chemotherapy	B cell lymphoma
LeukoSite/Ilex Oncology	LDP-03, huMAb to the leukocyte antigen CAMPATH	Chronic lymphocytic leukemia (CLL)
Center of Molecular Immunology	ior t6 (anti CD6, murine MAb) CTCL	Cancer
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Breast, ovarian
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Prostate, non-small cell lung, pancreatic, breast
Medarex	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Acute myelogenous leukemia (AML)
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Renal and colon
Medarex	MDX-11 (complement activating receptor (CAR) monoclonal antibody)	Ex vivo bone marrow purging in acute myelogenous leukemia (AML)
Medarex	MDX-22 (humanized bispecific antibody, MAb-conjugates) (complement cascade activators)	Acute myeloid leukemia
Cytogen	OV103 (Yttrium-90 labelled antibody)	Ovarian
Cytogen	OV103 (Yttrium-90 labelled antibody)	Prostate
Aronex Pharmaceuticals, Inc.	ATRAGEN®	non-Hodgkin's lymphoma
Glaxo Wellcome plc	3622W94 MAb that binds to EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas	non-small cell lung, prostate (adjuvant)
Genentech	Anti-VEGF, RhuMAb (inhibits angiogenesis)	Lung, breast, prostate, colorectal
Protein Design Labs	Zenapax (SMART Anti-Tac (IL-2 receptor) Ab, humanized)	Leukemia, lymphoma
Protein Design Labs	SMART M195 Ab, humanized	Acute promyelocytic leukemia
ImClone Systems	C225 (chimeric anti-EGFr monoclonal antibody) + taxol	Breast
ImClone Systems (licensed from RPR)	C225 (chimeric anti-EGFr monoclonal antibody) + doxorubicin	prostate
ImClone Systems	C225 (chimeric anti-EGFr monoclonal antibody) + adriamycin	prostate
ImClone Systems	BEC2 (anti-idiotypic MAb, mimics the GD_3 epitope)	Melanoma
Medarex	MDX-210 (humanized anti-HER-2 bispecific	Cancer

	antibody)	
Medarex	MDX-220 (bispecific for tumors that express TAG-72)	Lung, colon, prostate, ovarian, endometrial, pancreatic and gastric
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Prostate
Medarex/Merck KgaA	MDX-447 (humanized anti-EGF receptor bispecific antibody)	EGF receptor cancers (head & neck, prostate, lung, bladder, cervical, ovarian)
Medarex/Novartis	MDX-210 (humanized anti-HER-2 bispecific antibody)	Comb. Therapy with G-CSF for various cancers, esp. breast
IDEC	MELIMMUNE-2 (murine monoclonal antibody therapeutic vaccine)	Melanoma
IDEC	MELIMMUNE-1 (murine monoclonal antibody therapeutic vaccine)	Melanoma
Immunomedics, Inc.	CEACIDE™ (I-131)	Colorectal and other
NeoRx	Pretarget™ radioactive antibodies	non-Hodgkin's B cell lymphoma
Novopharm Biotech, Inc.	NovoMAb-G2 (pancarcinoma specific Ab)	Cancer
Techniclone Corporation/ Cambridge Antibody Technology	TNT (chimeric MAb to histone antigens)	Brain
Techniclone International/ Cambridge Antibody Technology	TNT (chimeric MAb to histone antigens)	Brain
Novopharm	Gliomab-H (Monoclonals – Humanized Abs)	Brain, melanomas, neuroblastomas
Genetics Institute/AHP	GNI-250 Mab	Colorectal
Merck KgaA	EMD-72000 (chimeric-EGF antagonist)	Cancer
Immunomedics	LymphoCide (humanized LL2 antibody)	non-Hodgkin's B-cell lymphoma
Immunex/AHP	CMA 676 (monoclonal antibody conjugate)	Acute myelogenous leukemia
Novopharm Biotech, Inc.	Monopharm-C	Colon, lung, pancreatic
Novopharm Biotech, Inc.	4B5 anti-idiotype Ab	Melanoma, small-cell lung
Center of Molecular Immunology	ior egf/r3 (anti EGF-R humanized Ab)	Radioimmunotherapy
Center of Molecular Immunology	ior c5 (murine MAb colorectal) for radioimmunotherapy	Colorectal
Creative BioMolecules/ Chiron	BABS (biosynthetic antibody binding site) Proteins	Breast cancer
ImClone Systems/Chugai	FLK-2 (monoclonal antibody to fetal liver kinase-2 (FLK-2))	Tumor-associated angiogenesis
ImmunoGen, Inc.	Humanized MAb/small-drug conjugate	Small-cell lung
Medarex, Inc.	MDX-260 bispecific, targets GD-2	Melanoma, glioma,

		neuroblastoma
Procyon Biopharma, Inc.	ANA Ab	Cancer
Protein Design Labs	SMART 1D10 Ab	B-cell lymphoma
Protein Design Labs/Novartis	SMART ABL 364 Ab	Breast, lung, colon
Immunomedics, Inc.	ImmuRAIT-CEA	Colorectal

Yet other types of chemotherapeutic agents which can be used according to the invention include Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorombucil, Cytarabine HCl, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Floxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o.p'-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-AMSA), Azacitidine, Erthropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate.

Chemotherapeutic agents which are currently in development or in use in a clinical setting are shown in the Table below.

Cancer Drugs in Development or on the Market			
Marketer	**Brand Name**	**Generic Name**	**Indication**
Abbott	TNP 470/AGM 1470	Fragyline	Anti-Angiogenesis in Cancer
Takeda	TNP 470/AGM 1470	Fragyline	Anti-Angiogenesis in Cancer
Scotia	Meglamine GLA	Meglamine GLA	Bladder Cancer
Medeva	Valstar	Valrubicin	Bladder Cancer - Refractory in situ carcinoma
Medeva	Valstar	Valrubicin	Bladder Cancer – Papillary Cancer
Rhone Poulenc	Gliadel Wafer	Carmustaine + Polifepr Osan	Brain Tumor
Warner Lambert	Undisclosed Cancer (b)	Undisclosed Cancer (b)	Cancer
Bristol Myers Squib	RAS Famesyl Transferase Inhibitor	RAS FamesylTransferase Inhibitor	Cancer
Novartis	MMI 270	MMI 270	Cancer
Bayer	BAY 12-9566	BAY 12-9566	Cancer
Merck	Famesyl Transferase Inhibitor	Famesyl Transferase Inhibitor	Cancer (Solid tumors –pancrease, colon, lung, breast)

Pfizer	PFE	MMP	Cancer, angiogenesis
Pfizer	PFE	Tyrosine Kinase	Cancer, angiogenesis
Lilly	MTA/LY 231514	MTA/LY 231514	Cancer Solid Tumors
Lilly	LY 264618/Lometexol	Lometexol	Cancer Solid Tumors
Scotia	Glamolec	LiGLA (lithium-gamma linolenate)	Cancer, pancreatic, breast, colon
Warner Lambert	CI-994	CI-994	Cancer, Solid Tumors / Leukemia
Schering AG	Angiogenesis inhibitor	Angiogenesis Inhibitor	Cancer / Cardio
Takeda	TNP-470	n/k	Malignant Tumor
Smithkline Beecham	Hycamtin	Topotecan	Metastatic Ovarian Cancer
Novartis	PKC 412	PKC 412	Multi-Drug Resistant Cancer
Novartis	Valspodar	PSC 833	Myeloid Leukemia/Ovarian Cancer
Immunex	Novantrone	Mitoxantrone	Pain related to hormone refractory prostate cancer.
Warner Lambert	Metaret	Suramin	Prostate
Genentech	Anti-VEGF	Anti-VEGF	Prostate / Breast / Colorectal / NSCL Cancer
British Biotech	Batimastat	Batimastat (BB94)	Pterygium
Eisai	E 7070	E 7070	Solid Tumors
Biochem Pharma	BCH-4556	BCH-4556	Solid Tumors
Sankyo	CS-682	CS-682	Solid Tumors
Agouron	AG2037	AG2037	Solid Tumors
IDEC Pharma	9-AC	9-AC	Solid Tumors
Agouron	VEGF/b-FGF Inhibitors	VEGF/b-FGF Inhibitors	Solid Tumors
Agouron	AG3340	AG3340	Solid Tumors / Macular Degen
Vertex	Incel	VX-710-	Solid Tumors - IV
Vertex	VX-853	VX-853	Solid Tumors - Oral
Zeneca	ZD 0101 (inj)	ZD 0101	Solid Tumors
Novartis	ISI 641	ISI 641	Solid Tumors
Novartis	ODN 698	ODN 698	Solid Tumors
Tanube Seiyaku	TA 2516	Marimistat	Solid Tumors
British Biotech	Marimastat	Marimastat (BB 2516)	Solid Tumors
Celltech	CDP 845	Aggrecanase Inhibitor	Solid Tumors / Breast Cancer
Chiroscience	D2163	D2163	Solid Tumors / Metastases
Warner Lambert	PD 183805	PD 183805	
Daiichi	DX8951f	DX8951f	Anti-Cancer
Daiichi	Lemonal DP 2202	Lemonal DP 2202	Anti-Cancer
Fujisawa	FK 317	FK 317	Anticancer Antibiotic
Chugai	Picibanil	OK-432	Antimalignant

			Tumor
Nycomed Amersham	AD 32/valrubicin	Valrubicin	Bladder Cancer-Refractory Insitu Carcinoma
Nycomed Amersham	Metastron	Strontium Derivative	Bone Cancer (adjunt therapy, Pain)
Schering Plough	Temodal	Temozolomide	Brain Tumours
Schering Plough	Temodal	Temozolonide	Brain Tumours
Liposome	Evacet	Doxorubicin, Liposomal	Breast Cancer
Nycomed Amersham	Yewtaxan	Paclitaxel	Breast Cancer Advanced, Ovarian Cancer Advanced
Bristol Myers Squib	Taxol	Paclitaxel	Breast Cancer Advanced, Ovarian Cancer Advanced, NSCLC
Roche	Xeloda	Capecitabine	Breast Cancer, Colorectal Cancer
Roche	Furtulon	Doxifluridine	Breast Cancer, Colorectal Cancer, Gastric Cancer
Pharmacia & Upjohn	Adriamycin	Doxorubicin	Breast Cancer, Leukemia
Ivax	Cyclopax	Paclitaxel, Oral	Breast/Ovarian Cancer
Rhone Poulenc	Oral Taxoid	Oral Taxoid	Broad Cancer
AHP	Novantrone	Mitoxantrone	Cancer
Sequus	SPI-077	Cisplatin, Stealth	Cancer
Hoechst	HMR 1275	Flavopiridol	Cancer
Pfizer	CP-358, 774	EGFR	Cancer
Pfizer	CP-609, 754	RAS Oncogene Inhibitor	Cancer
Bristol Myers Squib	BMS-182751	Oral Platinum	Cancer (Lung, Ovarian)
Bristol Myers Squib	UFT (Tegafur/Uracil)	UFT (Tegafur/Uracil)	Cancer Oral
Johnson & Johnson	Ergamisol	Levamisole	Cancer Therapy
Glaxo Wellcome	Eniluracil/776C85	5FU Enhancer	Cancer, Refractory Solid & Colorectal Cancer
Johnson & Johnson	Ergamisol	Levamisole	Colon Cancer
Rhone Poulenc	Campto	Irinotecan	Colorectal Cancer, Cervical Cancer
Pharmacia & Upjohn	Camptosar	Irinotecan	Colorectal Cancer, Cervical Cancer
Zeneca	Tomudex	Ralitrexed	Colorectal Cancer, Lung Cancer, Breast Cancer
Johnson & Johnson	Leustain	Cladribine	Hairy Cell Leukaemia
Ivax	Paxene	Paclitaxel	Kaposi Sarcoma
Sequus	Doxil	Doxorubicin, Liposomal	KS/Cancer
Sequus	Caelyx	Doxorubicin, Liposomal	KS/Cancer
Schering AG	Fludara	Fludarabine	Leukaemia
Pharmacia & Upjohn	Pharmorubicin	Epirubicin	Lung/Breast Cancer

Chiron	DepoCyt	DepoCyt	Neoplastic Meningitis
Zeneca	ZD1839	ZD 1839	Non Small Cell Lung Cancer, Pancreatic Cancer
BASF	LU 79553	Bis-Naphtalimide	Oncology
BASF	LU 103793	Dolastain	Oncology
Shering Plough	Caetyx	Doxorubicin-Liposome	Ovarian/Breast Cancer
Lilly	Gemzar	Gemcitabine	Pancreatic Cancer, Non Small Cell Lung Cancer, Breast, Bladder and Ovarian
Zeneca	ZD 0473/Anormed	ZD 0473/Anormed	Platinum based NSCL, ovarian etc.
Yamanouchi	YM 116	YM 116	Prostate Cancer
Nycomed Amersham	Seeds/I-125 Rapid St	Lodine Seeds	Prostate Cancer
Agouron	Cdk4/cdk2 inhibitors	cdk4/cdk2 inhibitors	Solid Tumors
Agouron	PARP inhibitors	PARP Inhibitors	Solid Tumors
Chiroscience	D4809	Dexifosamide	Solid Tumors
Bristol Myers Squib	UFT (Tegafur/Uracil)	UFT (Tegafur/Uracil)	Solid Tumors
Sankyo	Krestin	Krestin	Solid Tumors
Asta Medica	Ifex/Mesnex	Ifosamide	Solid Tumors
Bristol Meyers Squib	Ifex/Mesnex	Ifosamide	Solid Tumors
Bristol Myers Squib	Vumon	Teniposide	Solid Tumors
Bristol Myers Squib	Paraplatin	Carboplatin	Solid Tumors
Bristol Myers Squib	Plantinol	Cisplatin, Stealth	Solid Tumors
Bristol Myers Squib	Plantinol	Cisplatin	Solid Tumors
Bristol Myers Squib	Vepeside	Etoposide	Solid Tumors Melanoma
Zeneca	ZD 9331	ZD 9331	Solid Tumors, Advanced Colorectal
Chugai	Taxotere	Docetaxel	Solid Tumors, Breast Cancer
Rhone Poulenc	Taxotere	Docetaxel	Solid Tumors, Breast Cancer
Glaxo Wellcome	Prodrug of guanine arabinside	prodrug of arabinside	T Cell Leukemia/Lymphoma & B Cell Neoplasm
Bristol Myers Squib	Taxane Analog	Taxane Analog	Taxol follow up

A cancer vaccine may be selected from the group consisting of EGF, Anti-idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, peptide antigen vaccines, toxin/antigen vaccines,

MVA-based vaccine, PACIS, BCG vacine, TA-HPV, TA-CIN, DISC-virus and ImmuCyst/TheraCys. Biological response modifiers include interferon, poly IC, and lymphokines such as IL-2. Hormone replacement therapy includes tamoxifen alone or in combination with progesterone.

Another use of the described immunostimulatory nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated CpG nucleic acids are predominantly of a class called "Th1" which is most marked by a cellular immune response and is associated with IL-12 and IFNγ. The other major type of immune response is termed a Th2 immune response, which is associated with more of an antibody immune response and with the production of IL-4, IL-5 and IL-10. In general, it appears that allergic diseases are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the immunostimulatory nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an immunostimulatory nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to a subject to treat or prevent an allergy.

Nucleic acids containing unmethylated CpG motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines.

As described in detail in the following Example 12, oligonucleotides containing an unmethylated CpG motif (i.e. TCCATGACGTTCCTGACGTT; SEQ ID NO:10), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO:11) prevented the development of an inflammatory cellular infiltrate and eosinophilia in a murine model of asthma. Furthermore, the suppression of eosinophilic inflammation was associated with a suppression of a Th2 response and induction of a Th1 response.

For use in therapy, an effective amount of an appropriate immunostimulatory nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing the oligonucleotide to be taken up by the appropriate target cells (e.g., B-cells and monocytic cells). Preferred routes of administration include oral and transdermal (e.g., via a patch). Examples of other routes of administration include injection

(subcutaneous, intravenous, parenteral, intraperitoneal, intrathecal, etc.). The injection can be in a bolus or a continuous infusion.

A nucleic acid alone or as a nucleic acid delivery complex or in a standard delivery device (such as those described below) can be administered in conjunction with a pharmaceutically acceptable carrier. As used herein, the phrase "pharmaceutically acceptable carrier" is intended to include substances that can be coadministered with a nucleic acid or a nucleic acid delivery complex and allows the nucleic acid to perform its indicated function. Examples of such carriers include solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of such media for pharmaceutically active substances are well known in the art. Any other conventional carrier suitable for use with the nucleic acids falls within the scope of the instant invention.

The language "effective amount" of a nucleic acid molecule refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated CpG for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or fungal infection. An effective amount for use as a vaccine adjuvant could be that amount useful for boosting a subjects immune response to a vaccine. An "effective amount" for treating asthma can be that amount useful for redirecting a Th2 type of immune response that is associated with asthma to a Th1 type of response. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation. For any compound described herein a therapeutically effective amount can be initially determined from *in vivo* animal models or *in vitro* in comparison with oligonucleotides know to produce an immune response.

Subject doses of the compounds described herein typically range from about 0.1 μg to 100 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween. More typically local doses range from about 10 μg to 20 mg per administration, and most typically from about 50 μg to 1 mg, with 2 - 4 administrations being spaced hours, days or weeks apart. More typically, immune stimulant doses range from 1 μg to 100 mg per administration, and most typically 10μg to 10 mg, with daily or weekly administrations. Subject doses of the compounds described herein

for parenteral delivery, wherein the compounds are delivered without another therapeutic agent may be in the same range as mucosal dose or may be 5 to 10,000 times higher than the effective mucosal dose or for immune stimulant applications, and more typically 10 to 1,000 times higher, and most typically 20 to 100 times higher. More typically parenteral doses for these purposes range from about 10 μg to 500 mg per administration, and most typically from about 500 μg to 25 mg, with 2 - 4 administrations being spaced hours, days or weeks apart. In some embodiments, however, parenteral doses for these purposes may be used in a range of 5 to 10,000 times higher than the typical doses described above.

The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

For use in therapy, an effective amount of the nucleic acid can be administered to a subject by any mode that delivers the nucleic acid to a subject. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Some routes of administration include but are not limited to oral, intranasal, intratracheal, inhalation, ocular, vaginal, rectal, parenteral (e.g. intramuscular, intradermal, intravenous or subcutaneous injection) and direct injection.

For oral administration, the compounds (i.e., nucleic acids) can be delivered alone without any pharmaceutical carriers or formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. The term "pharmaceutically-acceptable carrier" means one or more compatible solid or liquid filler, dilutants or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules,

after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions.

Dragee cores may be provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray, from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or

insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions may also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

The nucleic acids may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular nucleic acids selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories. Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di-and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

The nucleic acid may be directly administered to the subject or may be administered in conjunction with a pharmaceutically acceptable carrier or a delivery vehicle. The nucleic acid and optionally other therapeutic agents may be administered alone (e.g. in saline or buffer) or using any delivery vehicles known in the art. One type of delivery vehicle is referred to herein as a nucleic acid delivery complex. A "nucleic acid delivery complex" shall mean a nucleic acid molecule associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. dendritic cell surfaces and/or increased cellular uptake by target cells).

Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes may be sufficiently stable *in vivo* to reduce significant uncoupling prior to internalization by the target cell. However, the complex may be cleavable under appropriate conditions within the cell so that the nucleic acid may be released in a functional form.

The nucleic acids may be delivered by non-invasive methods as described above. Non-invasive delivery of compounds is desirable for treatment of children, elderly, animals, and even adults and also to avoid the risk of needle-stick injury. Delivery vehicles for delivering compounds to mucosal surfaces have been described and include but are not limited to: Cochleates (Gould-Fogerite et al., 1994, 1996); Emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Mowat et al., 1993, Carlsson et al., 1991, Hu et., 1998, Morein et al., 1999); Liposomes (Childers et al., 1999, Michalek et al., 1989, 1992, de Haan 1995a, 1995b); Live bacterial vectors (e.g., *Salmonella, Escherichia coli, Bacillus calmatte-guerin, Shigella, Lactobacillus*) (Hone et al., 1996, Pouwels et al., 1998, Chatfield et al., 1993, Stover et al., 1991, Nugent et al., 1998); Live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex) (Gallichan et al., 1993, 1995, Moss et al., 1996, Nugent et al., 1998, Flexner et al., 1988, Morrow et al., 1999); Microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); nucleic acid vaccines (Fynan et al., 1993, Kuklin et al., 1997, Sasaki et al., 1998, Okada et al., 1997, Ishii et al., 1997); Polymers (e.g. carboxymethylcellulose, chitosan) (Hamajima et al., 1998, Jabbal-Gill et al., 1998); Polymer rings (Wyatt et al., 1998); Proteosomes (Vancott et al., 1998, Lowell et al., 1988, 1996, 1997); Sodium Fluoride (Hashi et al., 1998); Transgenic plants (Tacket et al., 1998, Mason et al., 1998, Haq et al., 1995); Virosomes (Gluck et al., 1992, Mengiardi et al., 1995, Cryz et al., 1998); Virus-like particles (Jiang et al., 1999, Leibl et al., 1998).

The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1: Effects of ODNs on B Cell Total RNA Synthesis and Cell Cycle

B cells were purified from spleens obtained from 6-12 wk old specific pathogen free DBA/2 or BXSB mice (bred in the University of Iowa animal care facility; no substantial strain differences were noted) that were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) ("B cells"). B cells contained fewer than 1% $CD4^+$ or $CD8^+$ cells. $8x10^4$ B cells were dispensed in triplicate into 96 well microtiter plates in 100 µl RPMI containing 10% FBS (heat inactivated to 65°C for 30 min.), 50 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamate. 20 µM ODN were added at the start of culture for 20 h at 37°C, cells pulsed with 1 µCi of ^3H uridine, and harvested and counted 4 hr later. Ig secreting B cells were enumerated using the ELISA spot assay after culture of whole spleen cells with ODN at 20 µM for 48 hr. Data, reported in Table 1, represent the stimulation index compared to cells cultured without ODN. ^3H thymidine incorporation assays showed similar results, but with some nonspecific inhibition by thymidine released from degraded ODN (Matson. S and A.M. Krieg (1992) Nonspecific suppression of ^3H-thymidine incorporation by control oligonucleotides. *Antisense Research and Development 2*:325).

Example 2: Effects of ODN on Production of IgM from B cells

Single cell suspensions from the spleens of freshly killed mice were treated with anti-Thyl, anti-CD4, and anti-CD8 and complement by the method of Leibson et al., *J. Exp. Med. 154*:1681 (1981)). Resting B cells (<02% T cell contamination) were isolated from the 63 - 70% band of a discontinuous Percoll gradient by the procedure of DeFranco et al, *J. Exp. Med. 155*:1523 (1982). These were cultured as described above in 30 µM ODN or 20 µg/ml LPS for 48 hr. The number of B cells actively secreting IgM was maximal at this time point, as determined by ELIspot assay (Klinman, D.M. et al. *J. Immunol. 144*:506 (1990)). In that assay, B cells were incubated for 6 hrs on anti-Ig coated microtiter plates. The Ig they produced (>99% IgM) was detected using phosphatase-labelled anti-Ig (Southern Biotechnology Associated, Birmingham, AL). The antibodies produced by individual B cells were visualized by addition of BCIP (Sigma Chemical Co., St. Louis MO) which forms an insoluble blue precipitate in the presence of phosphatase. The dilution of cells producing 20 - 40 spots/well was used to determine the total number of antibody-secreting B cells/sample. All assays were performed in triplicate (data reported in Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increases in response to CpG-ODN.

Example 3: B cell Stimulation by Bacterial DNA

DBA/2 B cells were cultured with no DNA or 50 μg/ml of a) Micrococcus lysodeikticus; b) NZB/N mouse spleen; and c) NFS/N mouse spleen genomic DNAs for 48 hours, then pulsed with ^{3}H thymidine for 4 hours prior to cell harvest. Duplicate DNA samples were digested with DNAse I for 30 minutes at 37 C prior to addition to cell cultures. E coli DNA also induced an 8.8 fold increase in the number of IgM secreting B cells by 48 hours using the ELISA-spot assay.

DBA/2 B cells were cultured with either no additive, 50 μg/ml LPS or the ODN 1; 1a; 4; or 4a at 20 uM. Cells were cultured and harvested at 4, 8, 24 and 48 hours. BXSB cells were cultured as in Example 1 with 5, 10, 20, 40 or 80 μM of ODN 1; 1a; 4; or 4a or LPS. In this experiment, wells with no ODN had 3833 cpm. Each experiment was performed at least three times with similar results. Standard deviations of the triplicate wells were <5%.

Example 4: Effects of ODN on Natural killer (NK) activity

10×10^{6} C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM CpG or non-CpG ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51}Cr release assay with YAC-1 and 2C11, two NK sensitive target cell lines (Ballas, Z. K. et al. (1993) *J. Immunol.* 150:17). Effector cells were added at various concentrations to 10^{4} ^{51}Cr-labeled target cells in V-bottom microtiter plates in 0.2 ml, and incubated in 5% CO_2 for 4 hr. at 37°C. Plates were then centrifuged, and an aliquot of the supernatant counted for radioactivity. Percent specific lysis was determined by calculating the ratio of the ^{51}Cr released in the presence of effector cells minus the ^{51}Cr released when the target cells are cultured alone, over the total counts released after cell lysis in 2% acetic acid minus the ^{51}Cr cpm released when the cells are cultured alone.

Example 5: In Vivo Studies with CpG Phosphorothioate ODN

Mice were weighed and injected IP with 0.25 ml of sterile PBS or the indicated phosphorothioate ODN dissolved in PBS. Twenty four hours later, spleen cells were harvested, washed, and stained for flow cytometry using phycoerythrin conjugated 6B2 to gate on B cells in conjunction with biotin conjugated anti Ly-6A/E or anti-Ia^d (Pharmingen, San Diego, CA) or anti-Bla-1 (Hardy, R.R. et al., *J. Exp. Med. 159*:1169 (1984). Two mice were studied for each condition and analyzed individually.

Example 6: Titration of Phosphorothioate ODN for B Cell Stimulation

B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the CpG ODN 1d and 3Db and then either pulsed after 20 hr with ^3H uridine or after 44 hr with ^3H thymidine before harvesting and determining cpm.

Example 7: Rescue of B Cells From Apoptosis

WEHI-231 cells (5×10^4/well) were cultured for 1 hr. at 37 C in the presence or absence of LPS or the control ODN 1a or the CpG ODN 1d and 3Db before addition of anti-IgM (1μ/ml). Cells were cultured for a further 20 hr. before a 4 hr. pulse with 2 μCi/well ^3H thymidine. In this experiment, cells with no ODN or anti-IgM gave 90.4×10^3 cpm of ^3H thymidine incorporation by addition of anti-IgM. The phosphodiester ODN shown in Table 1 gave similar protection, though with some nonspecific suppression due to ODN degradation. Each experiment was repeated at least 3 times with similar results.

Example 8: In Vivo Induction of Murine IL-6

DBA/2 female mice (2 mos. old) were injected IP with 500μg CpG or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each time point. IL-6 was measured by Elisa, and IL-6 concentration was calculated by comparison to a standard curve generated using recombinant IL-6. The sensitivity of the assay was 10 pg/ml. Levels were undetectable after 8 hr.

Example 9: Systemic Induction of Murine IL-6 Transcription

Mice and cell lines. DBA/2, BALB/c, and C3H/HeJ mice at 5-10 wk of age were used as a source of lymphocytes. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and bred and maintained under specific pathogen-free conditions in the University of Iowa Animal Care Unit. The mouse B cell line CH12.LX was kindly provided by Dr. G. Bishop (University of Iowa, Iowa City).

Cell preparation. Mice were killed by cervical dislocation. Single cell suspensions were prepared aseptically from the spleens from mice. T cell depleted mouse splenocytes

were prepared by using anti-Thy-1.2 and complement and centrifugation over lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) as described (Krieg, A. M. et al., (1989) A role for endogenous retroviral sequences in the regulation of lymphocyte activation. *J. Immunol.* 143:2448).

ODN and DNA. Phosphodiester oligonucleotides (O-ODN) and the backbone modified phosphorothioate oligonucleotides (S-ODN) were obtained from the DNA Core facility at the University of Iowa or from Operon Technologies (Alameda, CA). *E. coli* DNA (Strain B) and calf thymus DNA were purchased from Sigma (St. Louis, MO). All DNA and ODN were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. *E. coli* and calf thymus DNA were single stranded prior to use by boiling for 10 min. followed by cooling on ice for 5 min. For some experiments, *E. coli* and calf thymus DNA were digested with DNAse I (2U/μg of DNA) at 37°C for 2 hr in 1X SSC with 5mM MgCl2. To methylate the cytosine in CpG dinucleotides in *E. coli* DNA, *E. coli* DNA was treated with CpG methylase (M. *SssI*; 2U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at 37°C. Methylated DNA was purified as above. Efficiency of methylation was confirmed by *Hpa* II digestion followed by analysis by gel electrophoresis. All enzymes were purchased from New England Biolabs (Beverly, MA). LPS level in ODN was less than 12.5 ng/mg and *E. coli* and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

Cell Culture. All cells were cultured at 37°C in a 5% CO_2 humidified incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μg/ml), CpG or non-CpG phosphodiester ODN (O-ODN) (20 μM), phosphorothioate ODN (S-ODN) (0.5 μM), or *E. coli* or calf thymus DNA (50 μg/ml) at 37°C for 24 hr. (for IL-6 production) or 5 days (for IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with CpG O-ODN along with various concentrations (1-10 μg/ml) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat IgG1 mAb to *E. coli* β-galactosidase (hybridoma GL113; ATCC, Rockville, MD) (20) for 5 days. At the end of incubation, culture supernatant fractions were analyzed by ELISA as below.

In vivo induction of IL-6 and IgM. BALB/c mice were injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), *E. coli* DNA (200 μg/100 μl PBS/mouse), or CpG or non-CpG S-ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time points. Liver, spleen, thymus, and bone marrow were removed and RNA was prepared from

those organs using RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturers protocol.

ELISA. Flat-bottomed Immun 1 plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 100 µl/well of anti-mouse IL-6 mAb (MP5-20F3) (2 µg/ml) or anti-mouse IgM µ-chain specific (5 µg/ml; Sigma, St. Louis, MO) in carbonate-bicarbonate, pH 9.6 buffer (15nM Na_2CO_3, 35 mM $NaHCO_3$) overnight at 4°C. The plates were then washed with TPBS (0.5 mM $MgCl_2$-$6H_20$, 2.68 mM KCl, 1.47 mM KH_2P0_4, 0.14 M NaCl, 6.6 mM K_2HP0_4, 0.5% Tween 20) and blocked with 10% FCS in TPBS for 2 hr at room temperature and then washed again. Culture supernatants, mouse sera, recombinant mouse IL-6 (Pharmingen, San Diego, CA) or purified mouse IgM (Calbiochem, San Diego, CA) were appropriately diluted in 10% FCS and incubated in triplicate wells for 6 hr at room temperature. The plates were washed and 100 µl/well of biotinylated rat anti-mouse IL-6 monoclonal antibodies (MP5-32C11, Pharmingen, San Diego, CA) (1µg/ml in 10% FCS) or biotinylated anti-mouse Ig (Sigma, St. Louis, MO) were added and incubated for 45 min. at room temperature following washes with TPBS. Horseradish peroxidase (HRP) conjugated avidin (Bio-rad Laboratories, Hercules, CA) at 1:4000 dilution in 10% FCS (100 µl/well) was added and incubated at room temperature for 30 min. The plates were washed and developed with o-phenylendiamine dihydrochloride (OPD; Sigma, St. Louis MO) 0.05 M phosphate-citrate buffer, pH 5.0, for 30 min. The reaction was stopped with 0.67 N H_2SO_4 and plates were read on a microplate reader (Cambridge Technology, Inc., Watertown, MA) at 490-600 nm. The results are shown in Figures 1 and 2.

RT-PCR. A sense primer, an antisense primer, and an internal oligonucleotide probe for IL-6 were synthesized using published sequences (Montgomery, R.A. and M.S. Dallman (1991), Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (*J. Immunol.*) 147:554). cDNA synthesis and IL-6 PCR was done essentially as described by Montgomery and Dallman (Montgomery, R.A. and M.S. Dallman (1991), Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (*J. Immunol.*) 147:554) using RT-PCR reagents from Perkin-Elmer Corp. (Hayward, CA). Samples were analyzed after 30 cycles of amplification by gel electrophoresis followed by unblot analysis (Stoye, J.P. et al., (1991) DNA hybridization in dried gels with fragmented probes: an improvement over blotting techniques, *Techniques* 3:123). Briefly, the gel was hybridized at room temperature for 30 min. in denaturation buffer (0.05 M NaOH, 1.5M NaCl) followed by incubation for 30 min. in renaturation buffer (1.5 M NaCl, 1 M Tris, pH 8) and a 30 min. wash in double distilled water. The gel was dried and prehybridized at 47°C for 2 hr. hybridization buffer (5X SSPE, 0.1% SDS) containing 10 µg/ml denatured salmon sperm DNA. The gel was hybridized with $2x10^6$

cpm/ml γ-[^{32} P]ATP end-labeled internal oligonucleotide probe for IL-6 (5'CATTTCCACGATTTCCCA3') SEQ ID NO:56) overnight at 47°C, washed 4 times (2X SSC, 0.2% SDS) at room temperature and autoradiographed. The results are shown in Figure 3.

Cell Proliferation assay. DBA/2 mice spleen B cells ($5x10^4$ cells/100 µl/well) were treated with media, CpG or non-CpG S-ODN (0.5 µM) or O-ODN (20 µM) for 24 hr at 37°C. Cells were pulsed for the last four hr. with either [^{3}H] Thymidine or [^{3}H] Uridine (1 µ Ci/well). Amounts of [^{3}H] incorporated were measured using Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL).

Transfections and CAT assays. WEHI-231 cells (10^7 cells) were electroporated with 20 µg of control or human IL-6 promoter-CAT construct (kindly provided by S. Manolagas, Univ. of Arkansas) (Pottratz, S.T. et al., (1994) 17B-estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J. Clin. Invest.* 93:944) at 250 mV and 960 µF. Cells were stimulated with various concentrations or CpG or non-CpG ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J.Y. Sheen (1988) A single phase-extraction assay for chloramphenicol acetyl transferase activity. *Gene* 76:271) 16 hr. after transfection. The results are presented in Figure 5.

Example 10: Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by CpG Motifs

ODN were synthesized on an Applied Biosystems Inc. (Foster City, CA) model 380A, 380B, or 394 DNA synthesizer using standard procedures (Beacage and Caruthers (1981) Deoxynucleoside phosphoramidites-- A new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Letters 22, 1859-1862.). Phosphodiester ODN were synthesized using standard beta-cyanoethyl phosphoramidite chemistry. Phosphorothioate linkages were introduced by oxidizing the phosphate linkage with elemental sulfur instead of the standard iodine oxidation. The four common nucleoside phosphoramidites were purchased from Applied Biosystems. All phosphodiester and thioate containing ODN were deprotected by treatment with concentrated ammonia at 55°C for 12 hours. The ODN were purified by gel exclusion chromatography and lyophilized to dryness prior to use. Phosphorodithioate linkages were introduced by using deoxynucleoside S-(b-benzoylmercaptoethyl) pyrrolidino thiophosphoramidites (Wiesler, W.T. et al., (1993) In Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs- Synthesis and Properties, Agrawal, S. (ed.), Humana Press, 191-206.). Dithioate

containing ODN were deprotected by treatment with concentrated ammonia at 55°C for 12 hours followed by reverse phase HPLC purification.

In order to synthesize oligomers containing methylphosphonothioates or methylphosphonates as well as phosphodiesters at any desired internucleotide linkage, two different synthetic cycles were used. The major synthetic differences in the two cycles are the coupling reagent where dialkylaminomethylnucleoside phosphines are used and the oxidation reagents in the case of methylphosphonothioates. In order to synthesize either derivative, the condensation time has been increased for the dialkylaminomethylnucleoside phosphines due to the slower kinetics of coupling (Jager and Engels, (1984) Synthesis of deoxynucleoside methylphosphonates via a phosphonamidite approach. Tetrahedron Letters 24, 1437-1440). After the coupling step has been completed, the methylphosphinodiester is treated with the sulfurizing reagent (5% elemental sulfur, 100 millimolar N,N-diamethylaminopyridine in carbon disulfide/pyridine/triethylamine), four consecutive times for 450 seconds each to produce methylphosphonothioates. To produce methylphosphonate linkages, the methylphosphinodiester is treated with standard oxidizing reagent (0.1 M iodine in tetrahydrofuran/2,6-lutidine/water).

The silica gel bound oligomer was treated with distilled pyridine/concentrated ammonia, 1:1, (v/v) for four days at 4 degrees centigrade. The supernatant was dried in vacuo, dissolved in water and chromatographed on a G50/50 Sephadex column.

As used herein, O-ODN refers to ODN which are phosphodiester; S-ODN are completely phosphorothioate modified; S-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are phosphorothioate modified; S_2-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are phosphorodithioate modified; and MP-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with CpG dinucleotides indicated by underlining) include:

3D (5" GAGAACGCTGGACCTTCCAT), (SEQ ID NO:14);
3M (5' TCCATGTCGGTCCTGATGCT) , (SEQ ID NO:22);
5 (5' GGCGTTATTCCTGACTCGCC), (SEQ ID NO:57); and
6 (5' CCTACGTTGTATGCGCCCAGCT), (SEQ ID NO:58).

These sequences are representative of literally hundreds of CpG and non-CpG ODN that have been tested in the course of these studies.

Mice. DBA/2, or BXSB mice obtained from The Jackson Laboratory (Bar Harbor, ME), and maintained under specific pathogen-free conditions were used as a source of lymphocytes at 5-10 wk of age with essentially identical results.

Cell proliferation assay. For cell proliferation assays, mouse spleen cells ($5x10^4$ cells/100 µl/well) were cultured at 37°C in a 5% CO_2 humidified incubator in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (heated to 65°C for experiments with O-ODN, or 56°C for experiments using only modified ODN), 1.5 µM L-glutamine, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin for 24 hr or 48 hr as indicated. 1 µCi of ^3H uridine or thymidine (as indicated) was added to each well, and the cells harvested after an additional 4 hours of culture. Filters were counted by scintillation counting. Standard deviations of the triplicate wells were <5%. The results are presented in Figures 6 - 8.

Example 11: Induction of NK Activity

Phosphodiester ODN were purchased from Operon Technologies (Alameda, CA). Phosphorothioate ODN were purchased from the DNA core facility, University of Iowa, or from The Midland Certified Reagent Company (Midland TX). *E.coli* (strain B) DNA and calf thymus DNA were purchased from Sigma (St. Louis, MO). All DNA and ODN were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. The LPS level in ODN was less than 12.5 ng/mg and *E.coli* and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

Virus-free, 4-6 week old, DBA/2, C57BL/6 (B6) and congenitally athymic BALB/C mice were obtained on contract through the Veterans Affairs from the National Cancer Institute (Bethesda, MD). C57BL/6 SCID mice were bred in the SPF barrier facility at the University of Iowa Animal Care Unit.

Human peripheral mononuclear blood leukocytes (PBMC) were obtained as previously described (Ballas, Z.K. et al., (1990) *J. Allergy Clin. Immunol.* 85:453; Ballas, Z.K. and W. Rasmussen (1990) *J. Immunol.* 145:1039; Ballas, Z.K. and W. Rasmussen (1993) *J. Immunol.* 150;17). Human or murine cells were cultured at 5 x 10^6/well, at 37°C in a 5% CO_2 humidified atmosphere in 24-well plates (Ballas, Z.K. et al., (1990) *J. Allergy Clin. Immunol.* 85:453; Ballas, Z.K. and W. Rasmussen (1990) *J. Immunol* 145:1039; and Ballas, Z.K. and W. Rasmussen (1993) *J. Immunol,* 150:17), with medium alone or with CpG or non-CpG ODN at the indicated concentrations, or with *E.coli* or calf thymus (50 µg/ml) at 37°C for 24 hr. All cultures were harvested at 18 hr. and the cells were used as effectors in a standard 4 hr. ^{51}Cr-release assay against K562 (human) or YAC-1 (mouse) target cells as

previously described. For calculation of lytic units (LU), 1 LU was defined as the number of cells needed to effect 30% specific lysis. Where indicated, neutralizing antibodies against IFN-β (Lee Biomolecular, San Diego, CA) or IL-12 (C15.1, C15.6, C17.8, and C17.15; provided by Dr. Giorgio Trinchieri, The Wistar Institute, Philadelphia, PA) or their isotype controls were added at the initiation of cultures to a concentration of 10 μg/ml. For anti-IL-12 addition, 10 μg of each of the 4 MAB (or isotype controls) were added simultaneously. Recombinant human IL-2 was used at a concentration of 100 U/ml.

Example 12: Prevention of the Development of an Inflammatory Cellular Infiltrate and Eosinophilia in a Murine Model of Asthma

6-8 week old C56BL/6 mice (from The Jackson Laboratory, Bar Harbor, ME) were immunized with 5,000 Schistosoma mansoni eggs by intraperitoneal (i.p.) injection on days 0 and 7. Schistosoma mansoni eggs contain an antigen (Schistosoma mansoni egg antigen (SEA)) that induces a Th2 immune response (e.g. production of IgE antibody). IgE antibody production is known to be an important cause of asthma.

The immunized mice were then treated with oligonucleotides (30μg in 200μl saline by i.p. injection), which either contained an unmethylated CpG motif (i.e. TCCATGACGTTCCTGACGTT; SEQ ID NO:10) or did not (i.e. control, TCCATGAGCTTCCTGAGTCT; SEQ ID NO:11). Soluble SEA (10μg in 25μl of saline) was administered by intranasal instillation on days 14 and 21. Saline was used as a control.

Mice were sacrificed at various times after airway challenge. Whole lung lavage was performed to harvest airway and alveolar inflammatory cells. Cytokine levels were measured from lavage fluid by ELISA. RNA was isolated from whole lung for Northern analysis and RT-PCR studies using CsCl gradients. Lungs were inflated and perfused with 4% paraformaldehyde for histologic examination.

Figure 9 shows that when the mice are initially injected with the eggs i.p., and then inhale the egg antigen (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated CpG motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg antigen (open triangles).

Figure 10 shows that the same results are obtained when only eosinophils present in the lung lavage are measured. Eosinophils are the type of inflammatory cell most closely associated with asthma.

Figure 11 shows that when the mice are treated with a control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along

with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

Figure 12 shows that very low doses of oligonucleotide (< 10μg) can give this protection.

Figure 13 shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

Figure 14 shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect the cytokine response of the lung to production of Il-12, indicating a Th1 type of immune response.

Figure 15 shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune response.

Example 13: CpG Oligonucleotides Induce Human PBMC to Secrete Cytokines.

Human PBMC were prepared from whole blood by standard centrifugation over ficoll hypaque. Cells (5 X 10^5/ml) were cultured in 10% autologous serum in 96 well microtiter plates with CpG or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 μg/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the case of TNF-α or 24 hr. for the other cytokines before supernatant harvest and assay, measured by ELISA using Quantikine kits or reagents from R&D Systems (pg/ml) or cytokine ELISA kits from Biosource (for IL-12 assay). Assays were performed as per the manufacturer's instructions. Data are presented in Table 6 as the level of cytokine above that in wells with no added oligodeoxynucleotide.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

CLAIMS

1. A composition comprising:

an oligonucleotide comprising:

5' $Y_1N_1CGN_2Y_2$ 3'

wherein Y_1 and Y_2 are, independent of one another, nucleic acid molecules having between 1 and 10 nucleotides, and wherein Y_1 includes at least one modified internucleotide linkage and Y_2 includes at least one modified internucleotide linkage and wherein N_1 and N_2 are nucleic acid molecules, each independent of one another having between 0 and 20 nucleotides, but wherein N_1CGN_2 has at least 6 nucleotides in total and wherein the nucleotides of N_1CGN_2 have a phosphodiester backbone.

2. The composition of claim 1, wherein Y_1 and Y_2 each independently has between 3 and 8 nucleotides.

3. The composition of claim 1, wherein Y_1 is comprised of at least three Gs.

4. The composition of claim 1, wherein Y_1 is comprised of at least four Gs.

5. The composition of claim 1, wherein Y_1 is comprised of at least seven Gs.

6. The composition of claim 1, wherein Y_1 is comprised of all Gs.

7. The composition of claim 1, wherein Y_1 is selected from the group consisting of TCGTCG (SEQ ID NO:65), TCGTCGT (SEQ ID NO:66), and TCGTCGTT (SEQ ID NO:67).

8. The composition of claim 1, wherein Y_2 is comprised of at least two Gs.

9 The composition of claim 1, wherein Y_2 is comprised of at least four Gs.

10. The composition of claim 1, wherein Y_2 is comprised of at least seven Gs.

11. The composition of claim 1, wherein Y_2 is comprised of all Gs.

12. The composition of claim 1, wherein Y_1 includes at least two modified internucleotide linkages and Y_2 includes at least two modified internucleotide linkages.

13. The composition of claim 1, wherein Y_1 includes between two and five modified internucleotide linkages and Y_2 includes between two and five modified internucleotide linkages.

14. The composition of claim 1, wherein Y_1 has at least two modified internucleotide linkages and Y_2 has at least five modified internucleotide linkages.

15. The composition of claim 1, wherein Y_1 has five modified internucleotide linkages and Y_2 has two modified internucleotide linkages.

16. The composition of any one of claims 1-15, wherein the modified internucleotide linkage is a phosphorothioate modified linkage.

17. The composition of any one of claims 1-15, wherein the modified internucleotide linkage is a phosphorodithioate modified linkage.

18. The composition of any one of claims 1-15, wherein the modified internucleotide linkage is a p-ethoxy modified linkage.

19. The composition of any of claims 1-15, wherein the nucleotides of N_1CGN_2 form a palindrome.

20. The composition of claim 19, wherein the oligonucleotide has a sequence of nucleotides GGGGTCAACGTTGAGGGGGG (SEQ ID NO:12).

21. The composition of any of claims 1-15, wherein the nucleotides of N_1CGN_2 do not form a palindrome.

22. The composition of claim 1, further comprising a pharmaceutical carrier.

23. The composition of claim 1, wherein the composition is formulated in a delivery device.

24. The composition of claim 23, wherein the delivery device is selected from the group consisting of cationic lipids, cell permeating proteins, and sustained release devices.

25. The composition of claim 24, wherein the sustained release device is a biodegradable polymer.

26. The composition of claim 24, wherein the sustained release device is a microparticle.

27. A method for activating an NK cell, comprising contacting an NK cell with the composition of any one of claims 1-15.

28. A method for activating a dendritic cell, comprising contacting a dendritic cell with the composition of any one of claims 1-15.

29. A method for activating a lymphocyte, comprising contacting a lymphocyte with the composition of any one of claims 1-15.

30. A method for treating or preventing a cancer, comprising administering to a subject having or at risk of having cancer, an effective amount for treating or preventing the cancer of the composition of any one of claims 1-15.

31. The method of claim 30, wherein the cancer is selected from the group consisting of hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple

myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, and colon carcinoma.

32. A vaccine formulation, comprising the composition of any one of claims 1-15 in combination with an antigen.

33. A method for treating or preventing a viral or retroviral infection, comprising administering to a subject having or at risk of having a viral or retroviral infection, an effective amount for treating or preventing the viral or retroviral infection of the composition of any one of claims 1-15.

34. The method of claim 33, wherein the virus is caused by a hepatitis virus.

35. The method of claim 33, wherein the virus is caused by HIV.

36. The method of claim 33, wherein the virus is caused by hepatitis B.

37. The method of claim 33, wherein the virus is caused by hepatitis C.

38. The method of claim 33, wherein the virus is caused by herpes virus.

39. The method of claim 33, wherein the virus is caused by papillomavirus.

40. The method of claim 33, wherein the subject is a non-human subject.

41. The method of claim 40, wherein the virus is caused by a virus selected from the group consisting of *herpesviridae*, *retroviridae*, and *orthomyxoviridae*.

42. A method for treating or preventing a bacterial infection, comprising administering to a subject having or at risk of having a bacterial infection, an effective amount for treating or preventing the bacterial infection of the composition of any one of claims 1-15.

43. The method of claim 42, wherein the bacterial infection is due to a bacteria selected from the group consisting of *helicobacter pyloris*, *staphylococcus aureus*, *streptococcus*, *haemophilusm*, *enterabacter*, and clostridium..

44. The method of claim 42, wherein the subject is a non-human subject.

45. The method of claim 44, wherein the infection is due to a bacteria selected from the group consisting of *haemophilus*, *campylobacter*, *clostridium*, *E.coli*, *staphylococcus*, and *streptococcus*.

46. A method for treating or preventing a parasite infection, comprising administering to a subject having or at risk of having a parasite infection, an effective amount for treating or preventing the parasite infection of the composition of any one of claims 1-15.

47. The method of claim 46, wherein the parasite infection is due to plasmoium falciparum.

48. The method of claim 46, wherein the parasite infection is due to toxoplasma gondii.

49. A method for treating or preventing asthma, comprising administering to a subject having or at risk of having asthma, an effective amount for treating or preventing the asthma of the composition of any one of claims 1-15.

50. The method of claim 49, wherein the asthma is allergic asthma.

51. A method for treating or preventing allergy, comprising administering to a subject having or at risk of having allergy, an effective amount for treating or preventing the allergy of the composition of any one of claims 1-15.

52. A method for treating or preventing an immune deficiency, comprising administering to a subject having or at risk of an immune deficiency, an effective amount for treating or preventing the immune deficiency of the composition of any one of claims 1-15.

53. The composition of any one of claims 1-15 further comprising a second immunostimulatory nucleic acid having an unmethylated CG dinucleotide, wherein the second immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a different sequence than the oligonucleotide comprising 5' $Y_1N_1CGN_2Y_2$ 3'.

54. The composition of claim 53, wherein the second immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a completely phosphodiester backbone.

55. The composition of claim 53, wherein the second immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a modified backbone.

56. The composition of claim 55, wherein the modified backbone has internucleotide linkages selected from the group consisting of phosphorothioate, phosphorodithioate, and p-ethoxy.

57. The composition of claim 53, wherein the second immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a formula comprising:

5' $X_1X_2CGX_3X_4$ 3'

wherein X_1, X_2, X_3 and X_4 are nucleotides.

58. The composition of claim 57, wherein the second immunostimulatory nucleic acid sequence includes at least the following formula:

5' $TCNTX_1X_2CGX_3X_4$ 3'

wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides, wherein at least one nucleotide has a modified internucleotide linkage, and wherein the nucleic acid has less than or equal to 100 nucleotides.

59. The composition of claim 58, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpG.

60. The composition of claim 58, wherein X_1X_2 are GpA and X_3X_4 are TpT.

61. The composition of claim 57, wherein the immunostimulatory nucleic acid sequence includes at least one of the following sequences:

ATCGACTCTCGAGCGTTCTC (SEQ ID NO:18)
TCCATGTCGGTCCTGCTGAT (SEQ ID NO:35)
TCCATGTCGGTZCTGATGCT (SEQ ID NO:34)
ATCGACTCTCGAGCGTTZTC (SEQ ID NO:21)
TCCATGTCGGTCCTGATGCT (SEQ ID NO:31)
GGGGTCAACGTTGAGGGGGG (SEQ ID NO:12)
TCCATGACGGTCCTGATGCT (SEQ ID NO:40)
TCCATGGCGGTCCTGATGCT (SEQ ID NO:39)
TCCATGACGTTCCTGATGCT (SEQ ID NO:44)
TCCATGTCGTTCCTGATGCT (SEQ ID NO:43)
GGGGTCAGTCTTGACGGGG (SEQ ID NO:50)
TCCATGTCGCTCCTGATGCT (SEQ ID NO:42)
TCCATGTCGATCCTGATGCT (SEQ ID NO:41)
TCCATGCCGGTCCTGATGCT (SEQ ID NO:38)
TCCATAACGTTCCTGATGCT (SEQ ID NO:45)
TCCATGACGTCCCTGATGCT (SEQ ID NO:46)
TCCATCACGTGCCTGATGCT (SEQ ID NO:47), and
TCCATGACGTTCCTGACGTT (SEQ ID NO:10).

62. A pharmaceutical composition, comprising:

a composition of at least two oligonucleotides as claimed in any one of claims 1-15, wherein the at least two oligonucleotides have different sequences from one another and a pharmaceutically acceptable carrier.

63. A method for inducing a TH1 immune response, comprising,

administering to a subject a composition of any one of claims 1-15 in an effective amount to produce a TH1 immune response.

64. A method of treating or preventing a cancer, comprising administering to a subject having or at risk of having cancer, an effective amount for treating or preventing the cancer the composition of any one of claims 1-15 and a second immunostimulatory nucleic acid having an unmethylated CG dinucleotide, wherein the second immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a different sequence than the oligonucleotide comprising 5' $Y_1N_1CGN_2Y_2$ 3'.

65. The method of claim 64, wherein the second immunostimulatory nucleic acid has a formula comprising:

5' $X_1X_2CGX_3X_4$ 3'

wherein X_1, X_2, X_3 and X_4 are nucleotides.

66. The method of claim 65, wherein the second immunostimulatory nucleic acid sequence includes at least the following formula:

5' $TCNTX_1X_2CGX_3X_4$ 3'

- wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides, wherein at least one nucleotide has a modified internucleotide linkage, and wherein the nucleic acid has less than or equal to 100 nucleotides.

67. A method of treating or preventing an infectious disease comprising administering to a subject having or at risk of having an infectious disease, an effective amount for treating or preventing the infectious disease the composition of any one of claims 1-15 and an immunostimulatory nucleic acid having an unmethylated CG dinucleotide, wherein the immunostimulatory nucleic acid having an unmethylated CG dinucleotide has a different sequence than the oligonucleotide comprising 5' $Y_1N_1CGN_2Y_2$ 3'.

68. The method of claim 67, wherein the immunostimulatory nucleic acid has a formula comprising:

5' $X_1X_2CGX_3X_4$ 3'

wherein X_1, X_2, X_3 and X_4 are nucleotides.

69. The method of claim 68, wherein the immunostimulatory nucleic acid sequence includes at least the following formula:

5' $TCNTX_1X_2CGX_3X_4$ 3'

wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides, wherein at least one nucleotide has a modified internucleotide linkage, and wherein the nucleic acid has less than or equal to 100 nucleotides.

70. The method of claim 67, wherein the infectious disease is caused by a viral or retroviral infection.

71. The method of claim 67, wherein the infectious disease is caused by a bacteria.

72. The method of claim 67, wherein the infectious disease is caused by a parasite.

73. The composition of claim 1, further comprising a cancer medicament.

74. The composition of claim 1, further comprising an antibody.

ABSTRACT

Nucleic acids containing unmethylated CpG dinucleotides and therapeutic utilities based on their ability to stimulate an immune response in a subject are disclosed.

FIGURE 1A

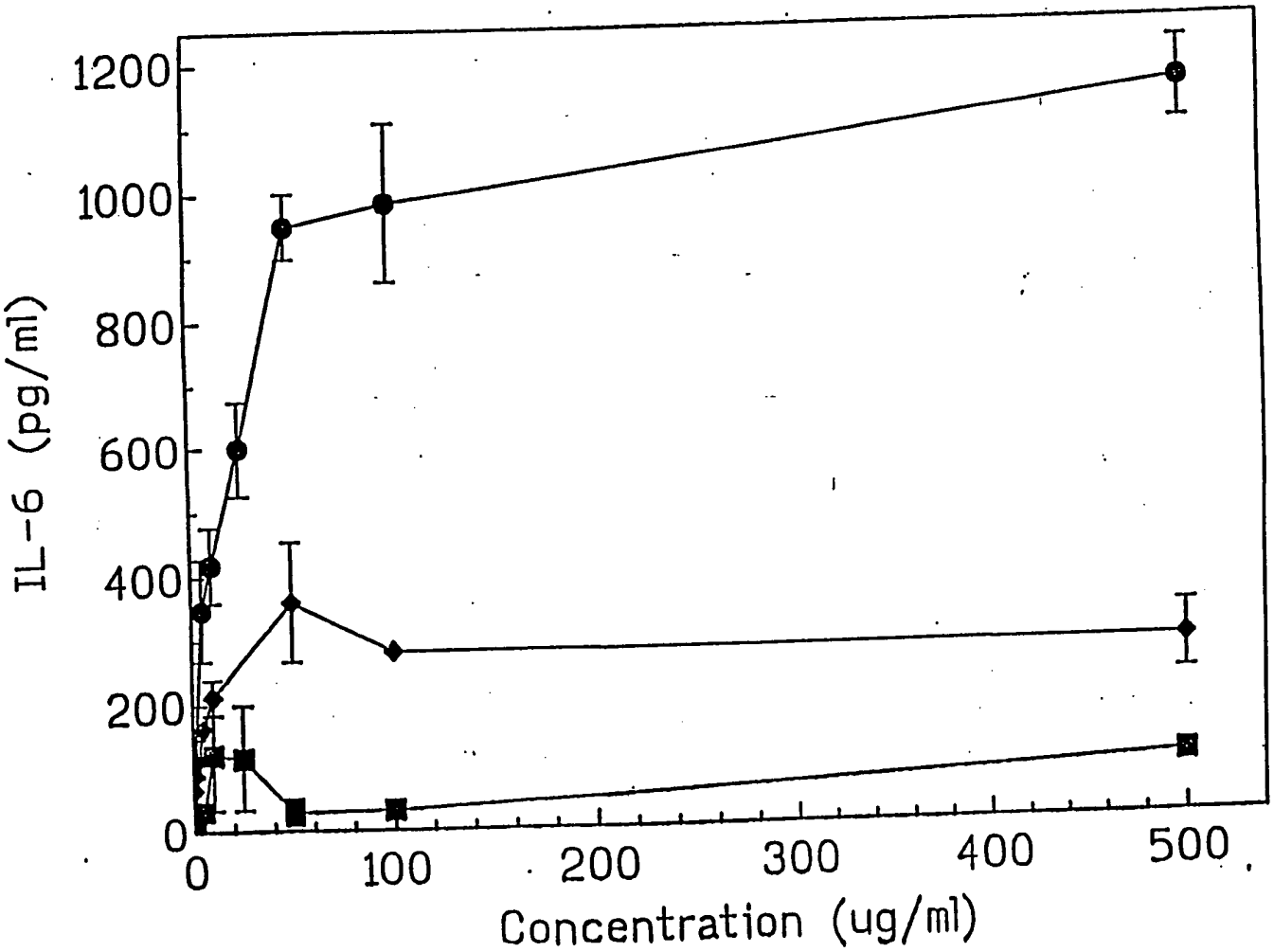

1200
1000
800
600
400
200
0
IL-6 (pg/ml)
0
100
200
300
400
500
Concentration (ug/ml)

FIGURE 1B

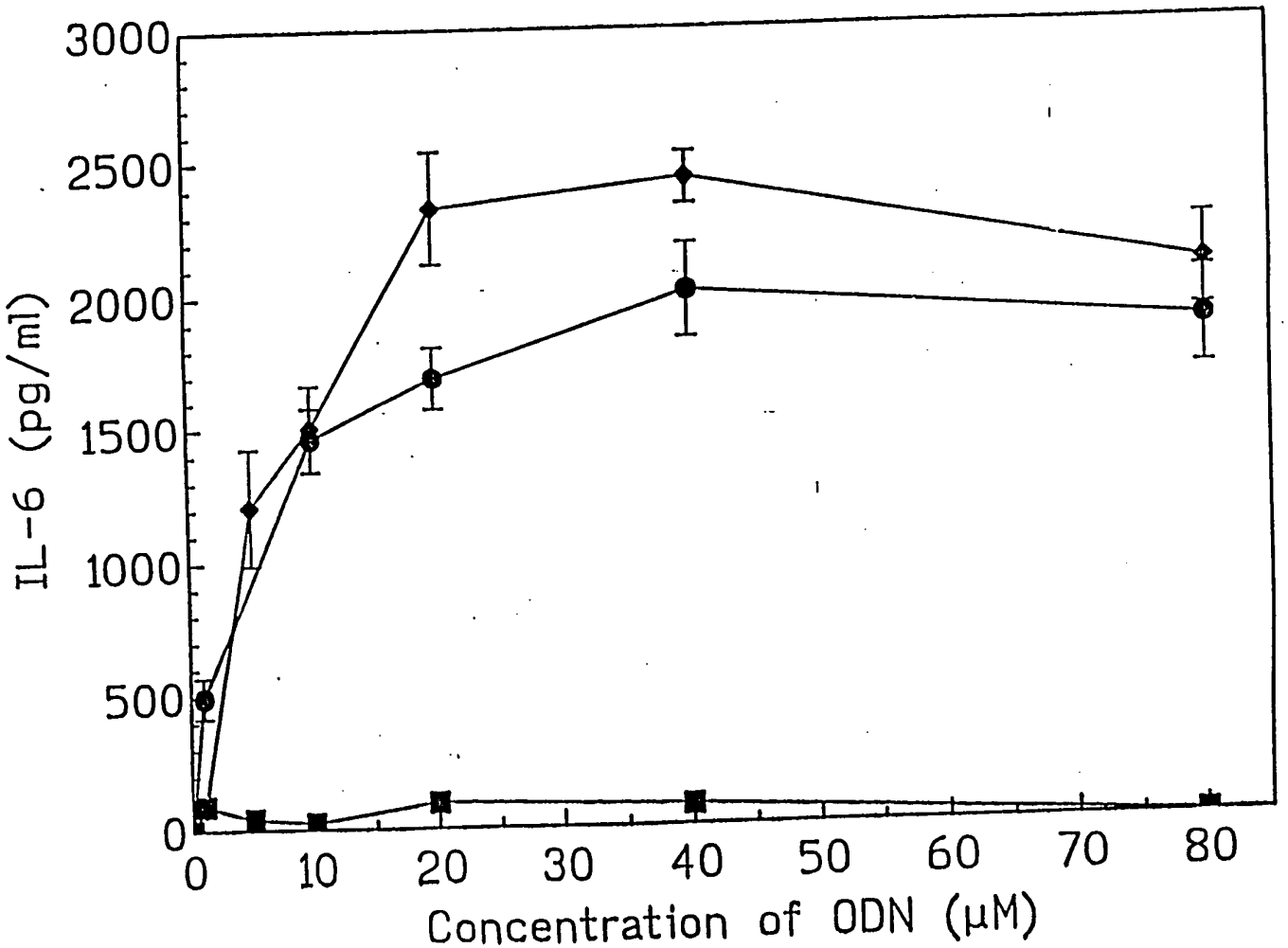

3000
2500
2000
1500
1000
500
0
IL-6 (pg/ml)
0
10
20
30
40
50
60
70
80
Concentration of ODN (μM)

FIGURE 1C

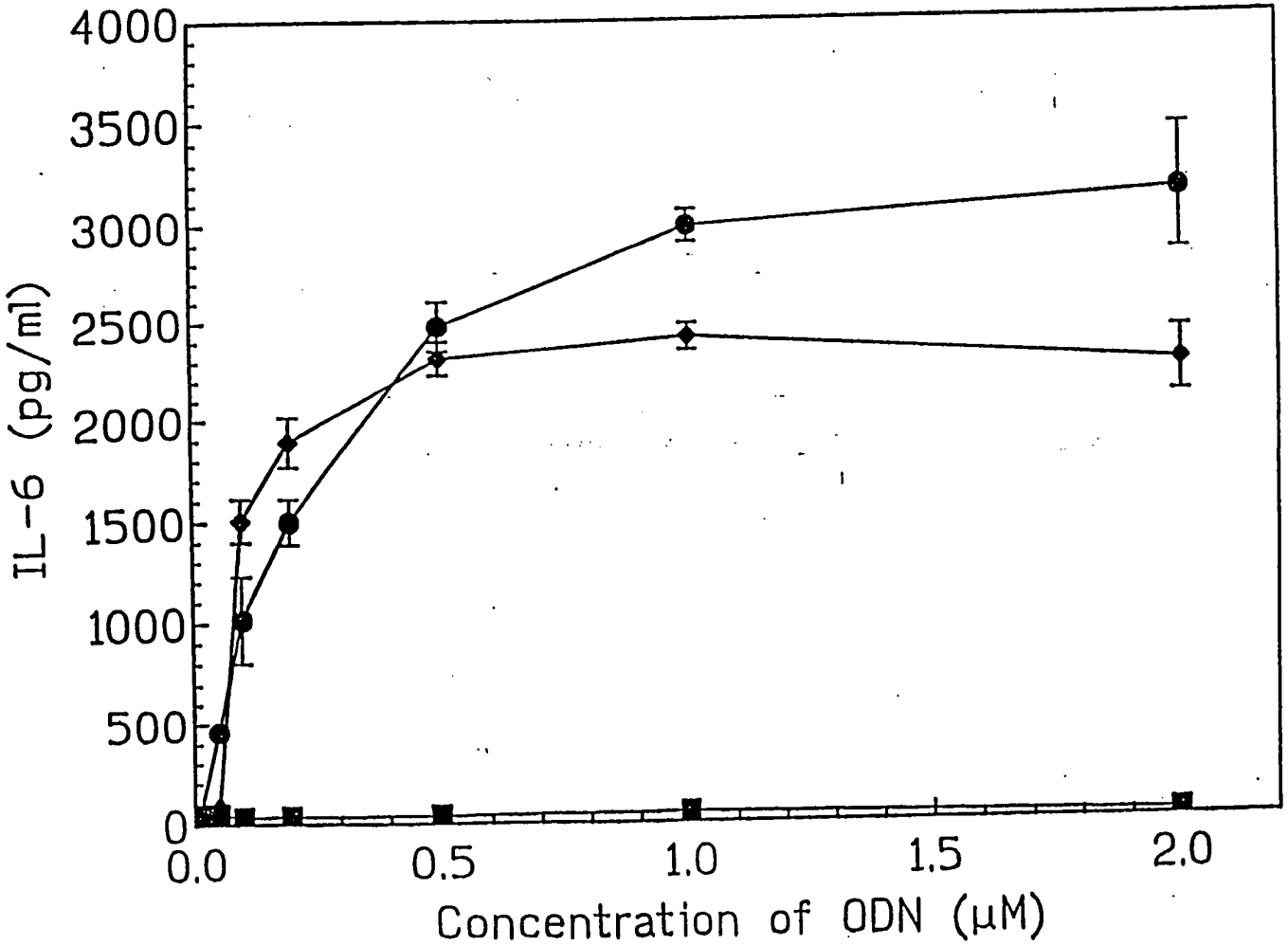
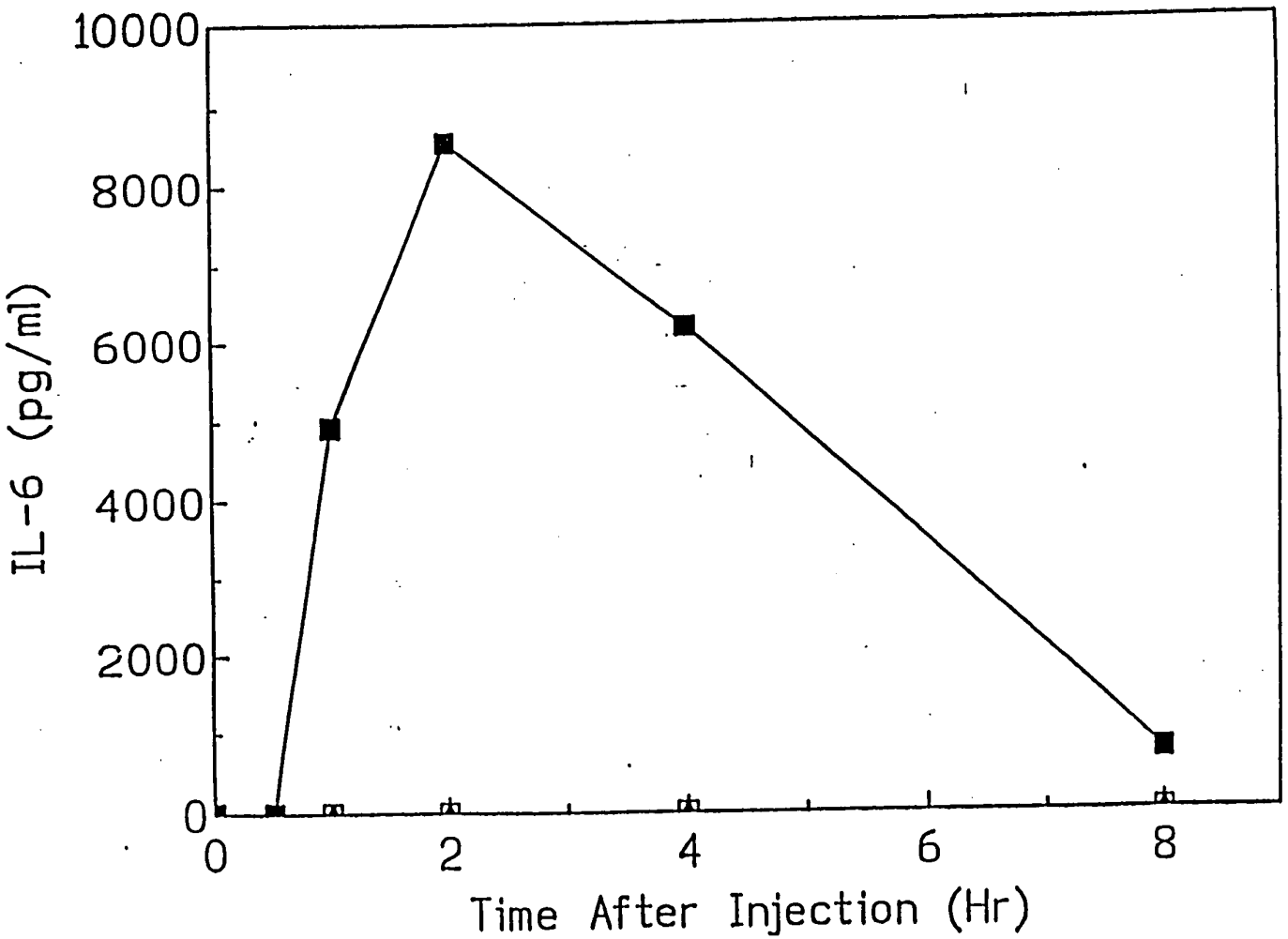

4000
3500
3000
2500
2000
1500
1000
500
0
IL-6 (pg/ml)
0.0
0.5
1.0
1.5
2.0
Concentration of ODN (μM)

FIGURE 2


10000
8000
6000
4000
2000
0
IL-6 (pg/ml)
0
2
4
6
8
Time After Injection (Hr)

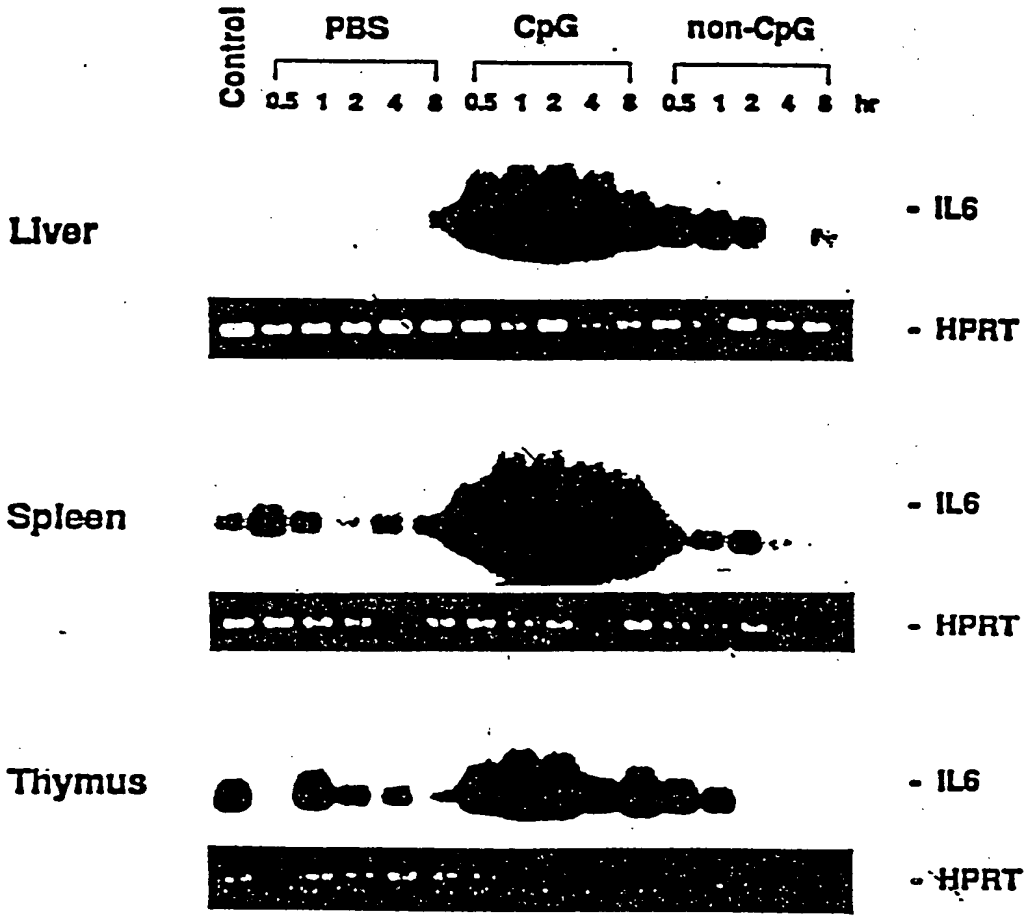

Control
PBS
CpG
non-CpG
0.5 1 2 4 8 0.5 1 2 4 8 0.5 1 2 4 8 hr
Liver
- IL6
- HPRT
Spleen
- IL6
- HPRT
Thymus
- IL6
- HPRT

FIGURE 3

FIGURE : 4A

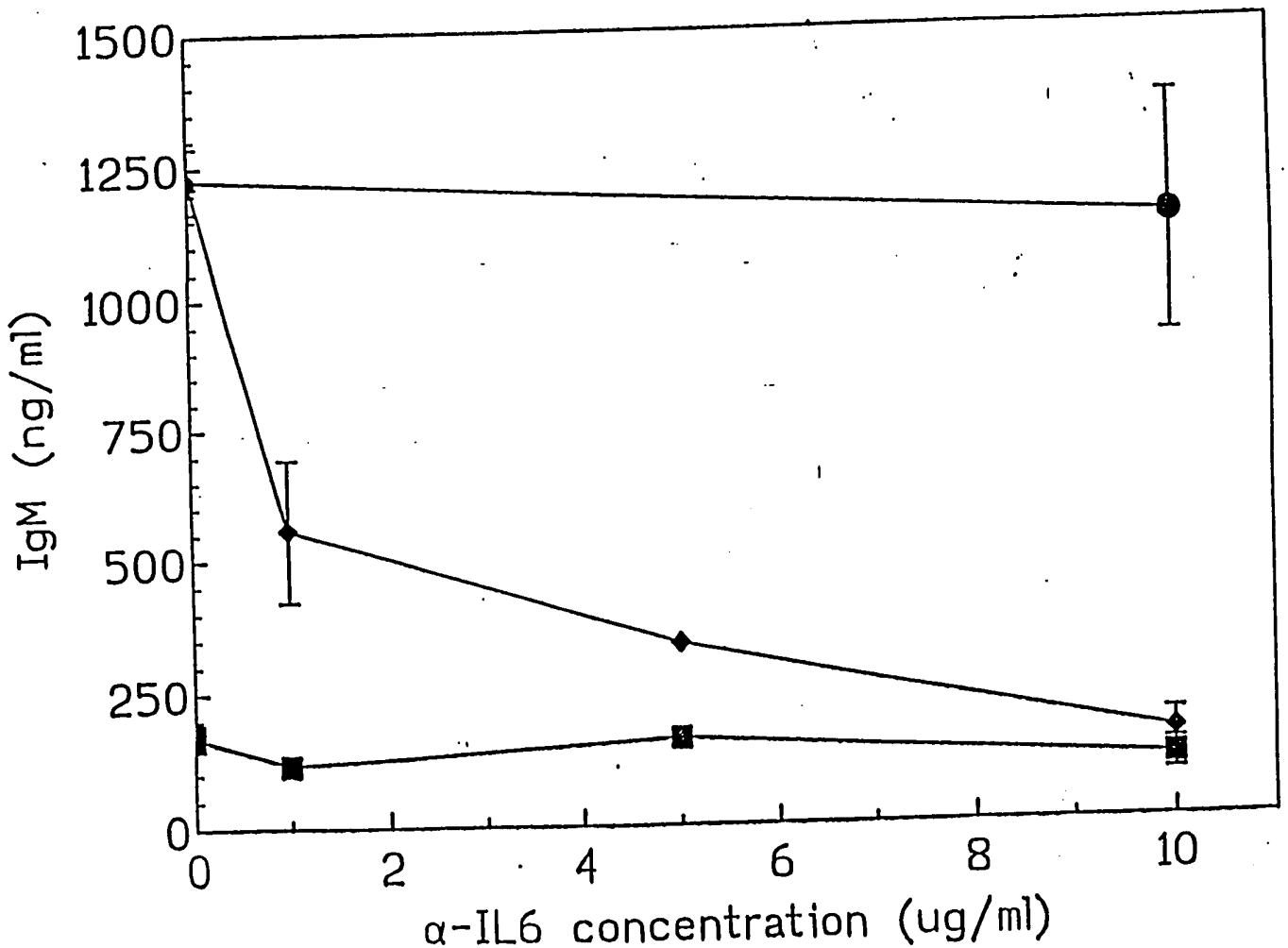
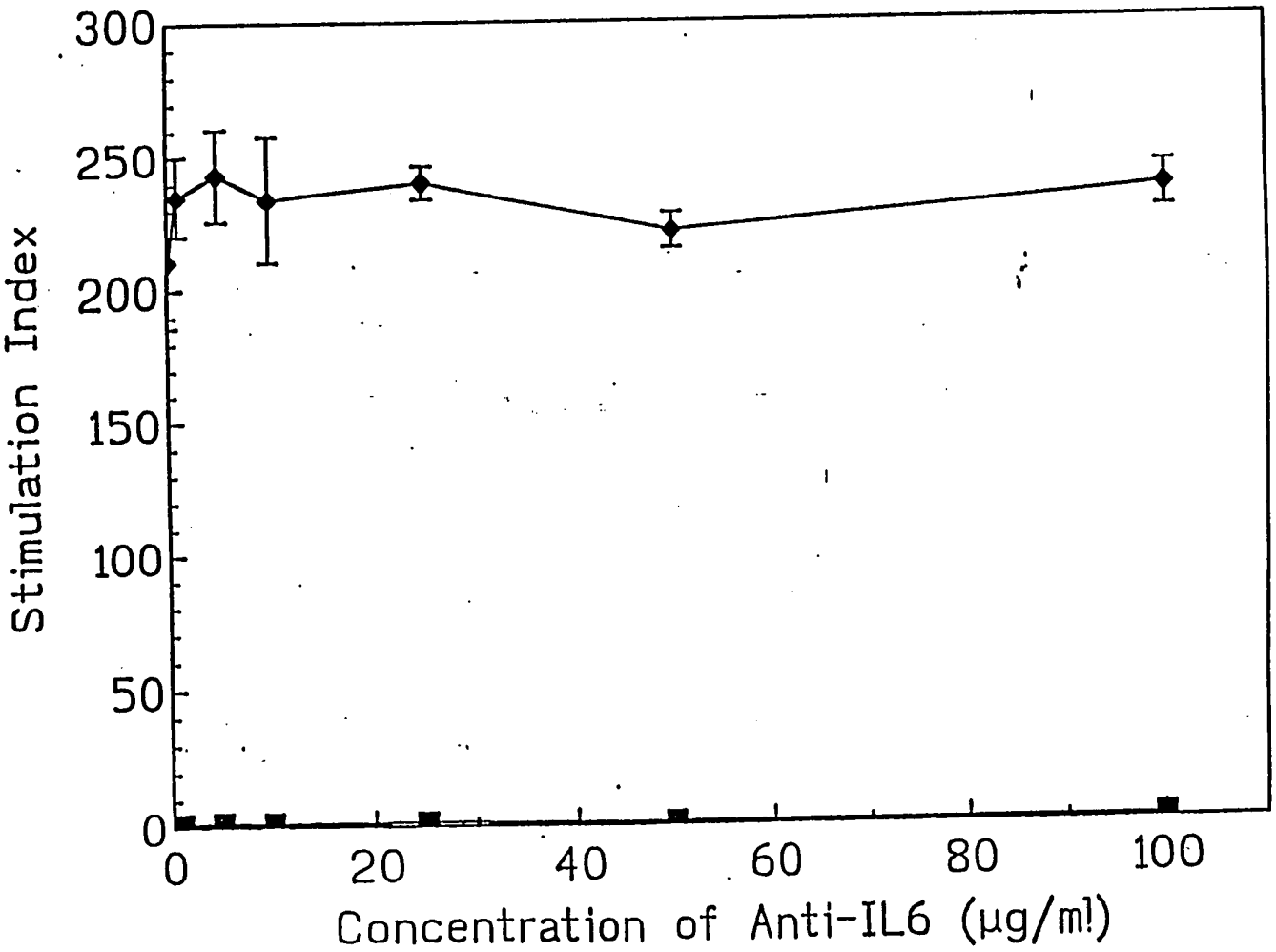

1500
1250
1000
750
500
250
0
IgM (ng/ml)
0
2
4
6
8
10
α-IL6 concentration (ug/ml)

FIGURE 4B


300
250
200
150
100
50
0
Stimulation Index
0
20
40
60
80
100
Concentration of Anti-IL6 (µg/ml)

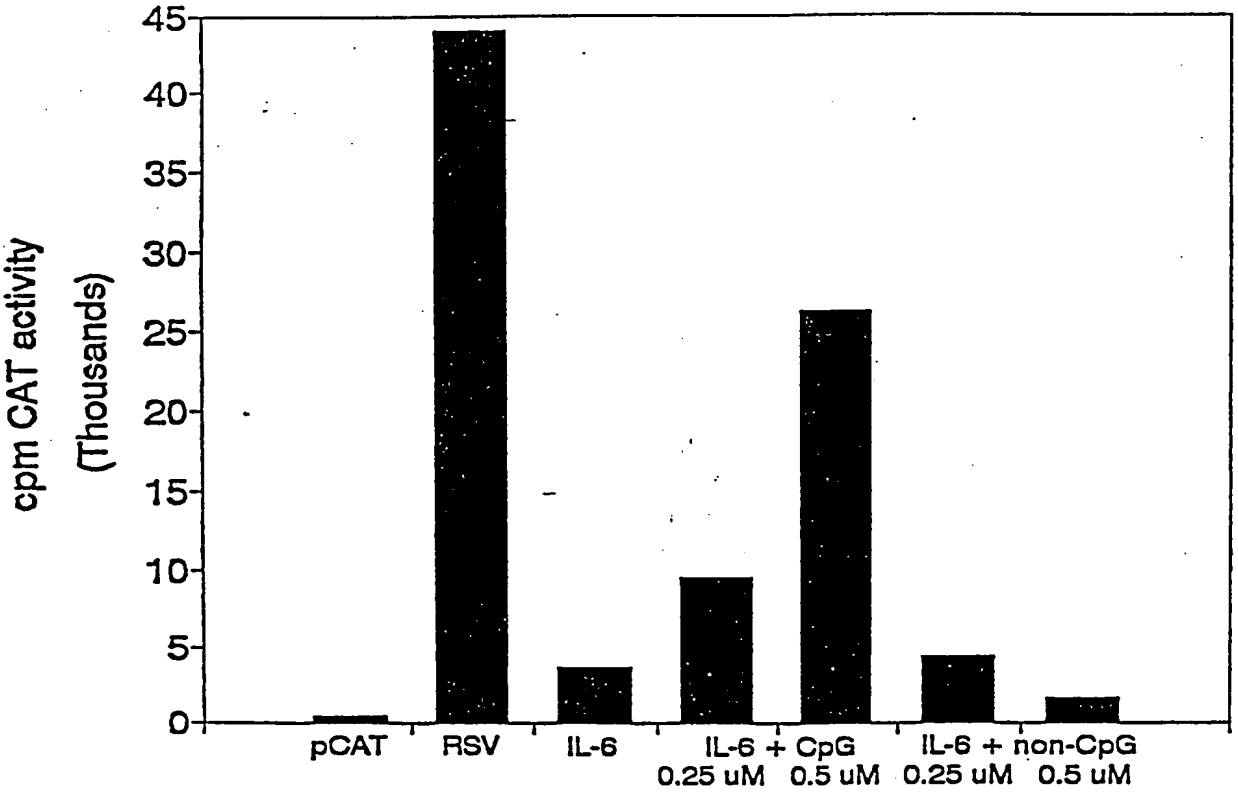

cpm CAT activity
(Thousands)
45
40
35
30
25
20
15
10
5
0
pCAT
RSV
IL-6
IL-6 + CpG
0.25 uM
0.5 uM
IL-6 + non-CpG
0.25 uM
0.5 uM

FIGURE 5

FIGURE 6

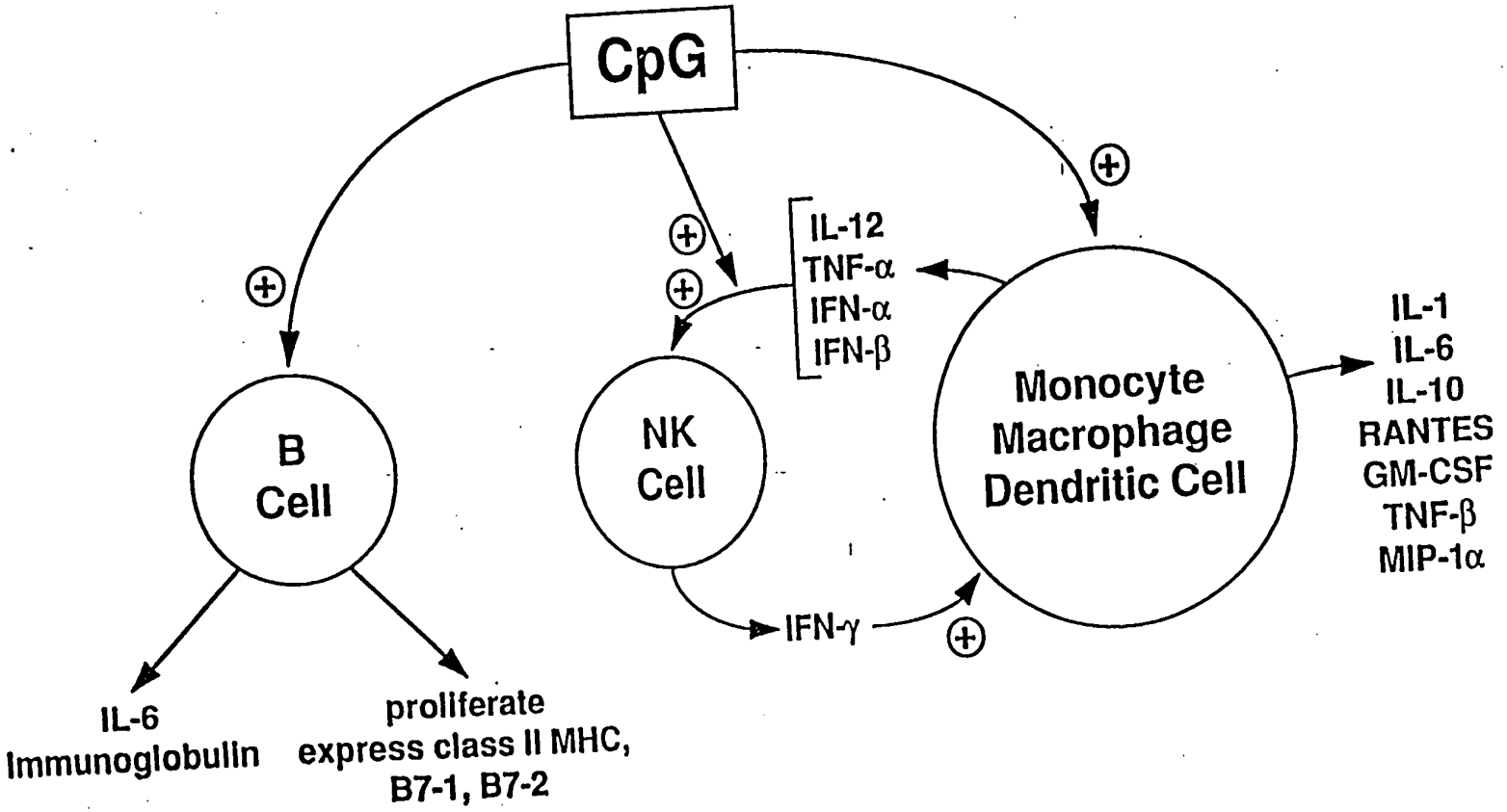

CpG
IL-12
TNF-α
IFN-α
IFN-β
B Cell
NK Cell
Monocyte Macrophage Dendritic Cell
IL-1
IL-6
IL-10
RANTES
GM-CSF
TNF-β
MIP-1α
IFN-γ
IL-6
Immunoglobulin
proliferate
express class II MHC,
B7-1, B7-2

FIGURE 7

Timing of NFκB Activation in Monocytes treated with E. coli DNA

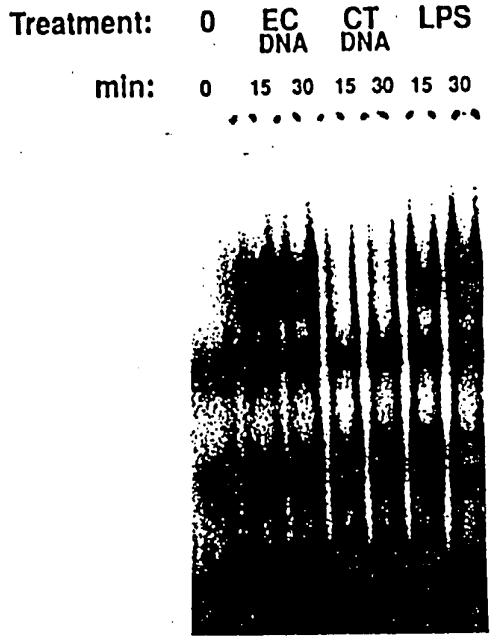


FIGURE 8A

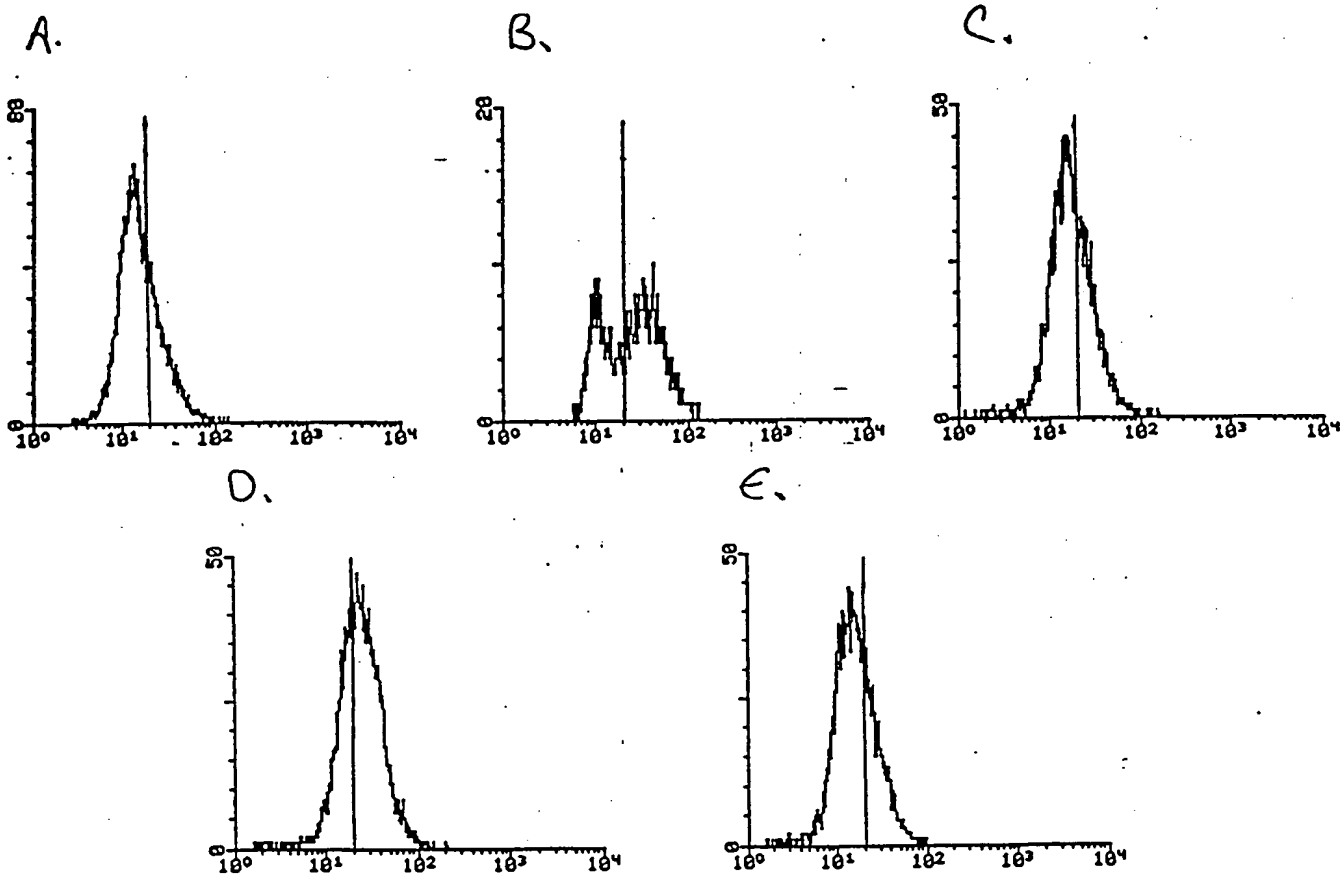


FIGURE 8B

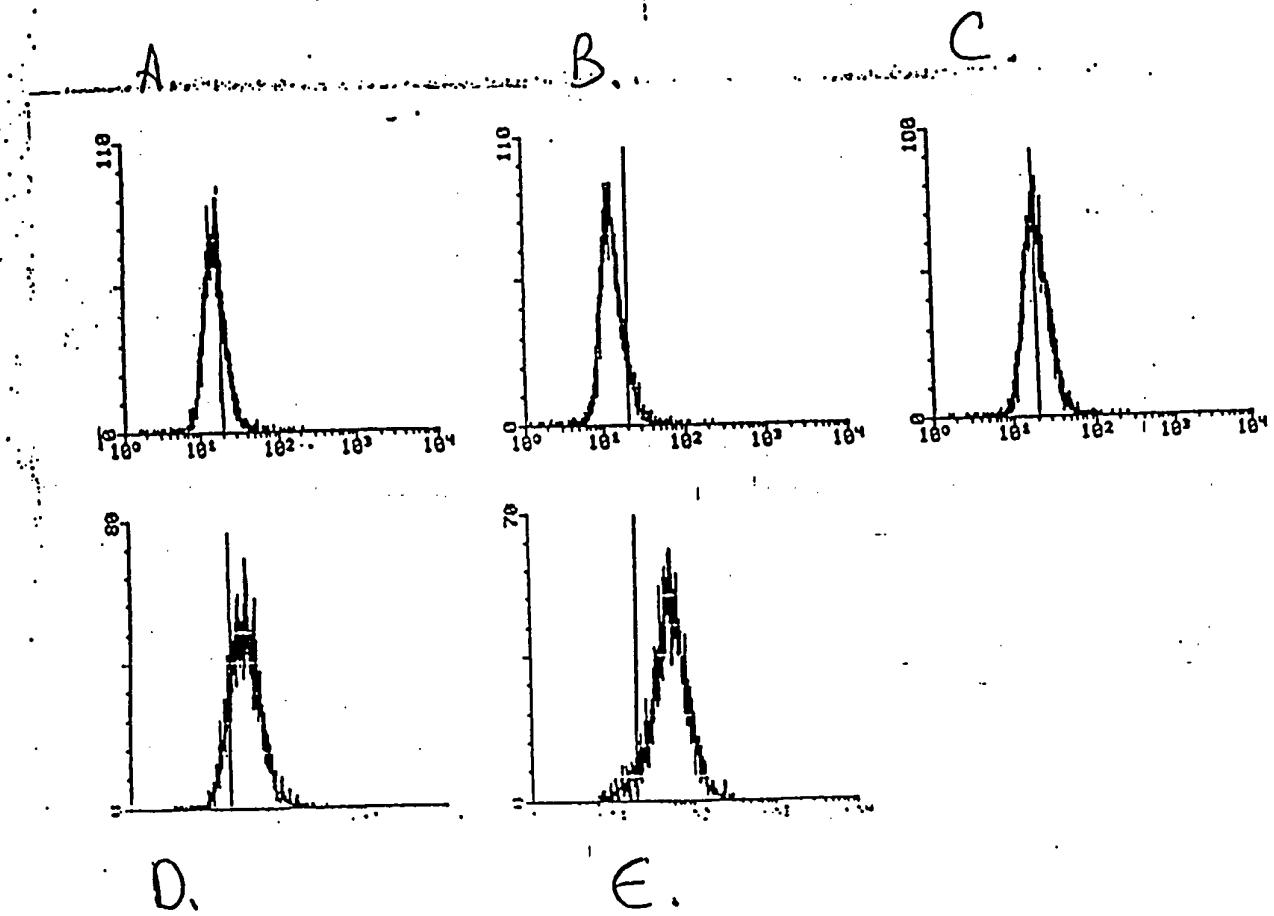


FIGURE 9

Effect of CpG and Airway Exposure on Lung Lavage Cell Count

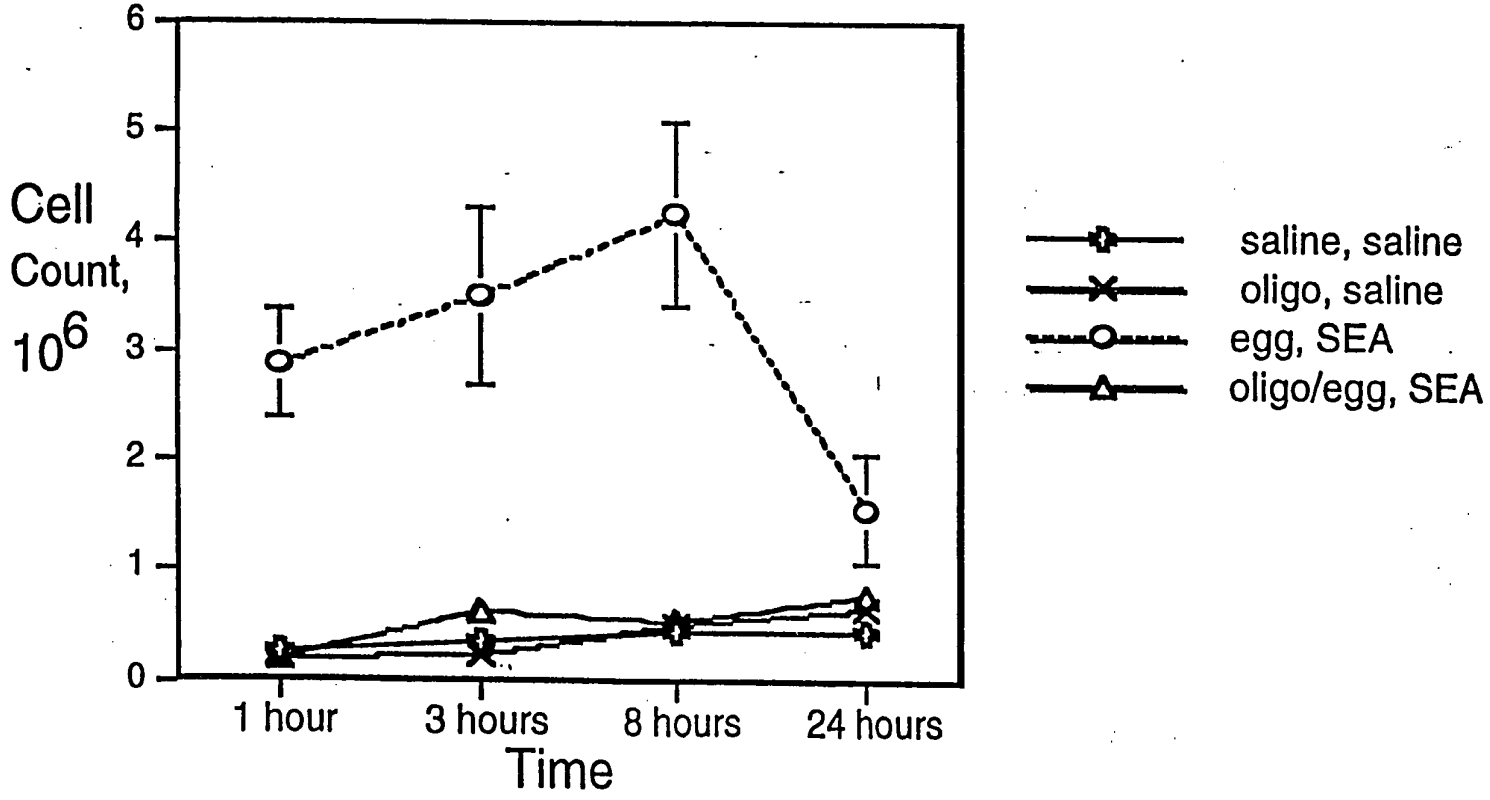


FIGURE 10

Effect of CpG and Airway Exposure on Lung Lavage Eosinophil Count

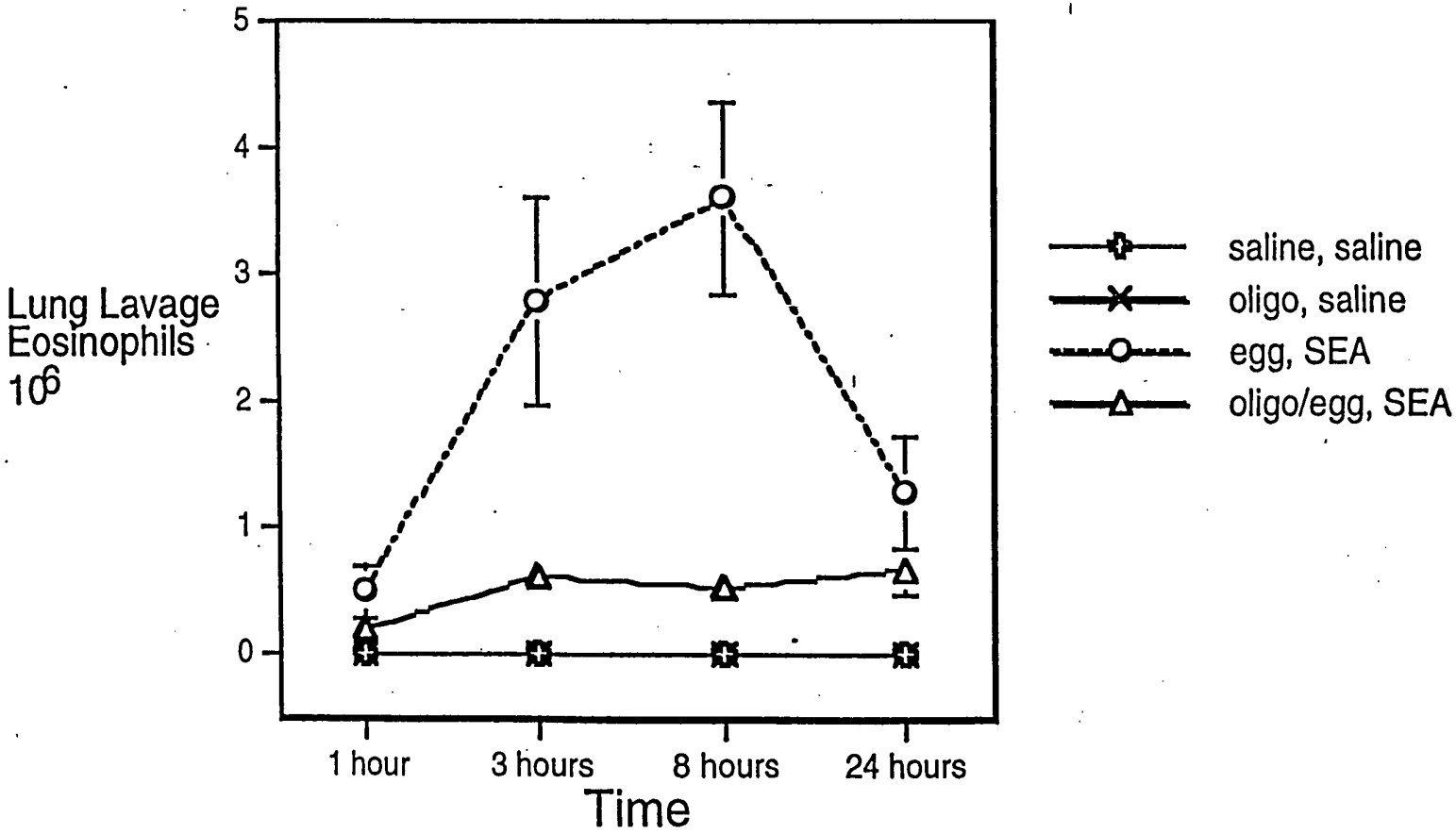


FIGURE 11

Effect of CpG and Airway Exposure on Lung Lavage Differential

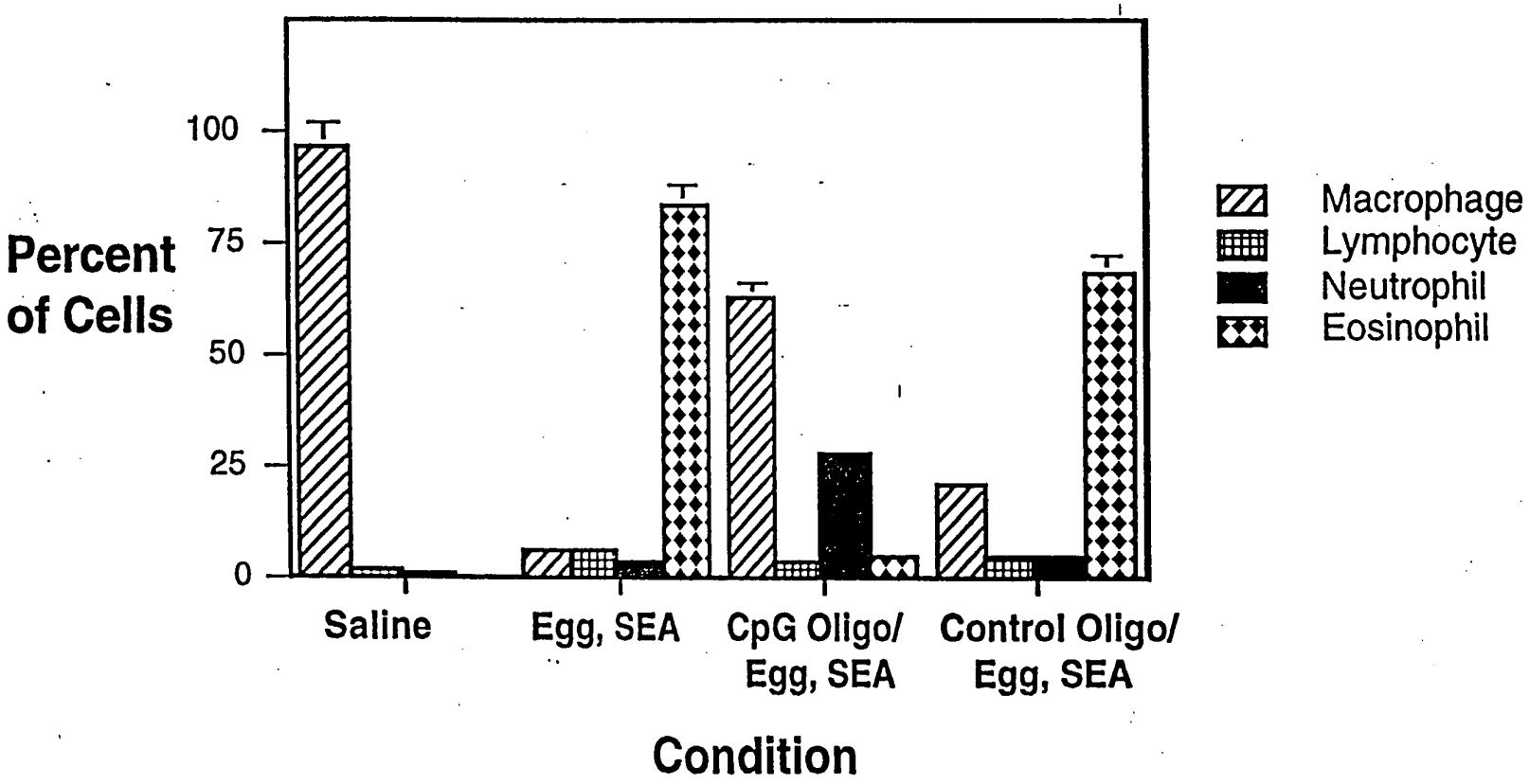


FIGURE 12

Effect of Oligonucleotide Dose on Total and Eosinophil Cell Counts

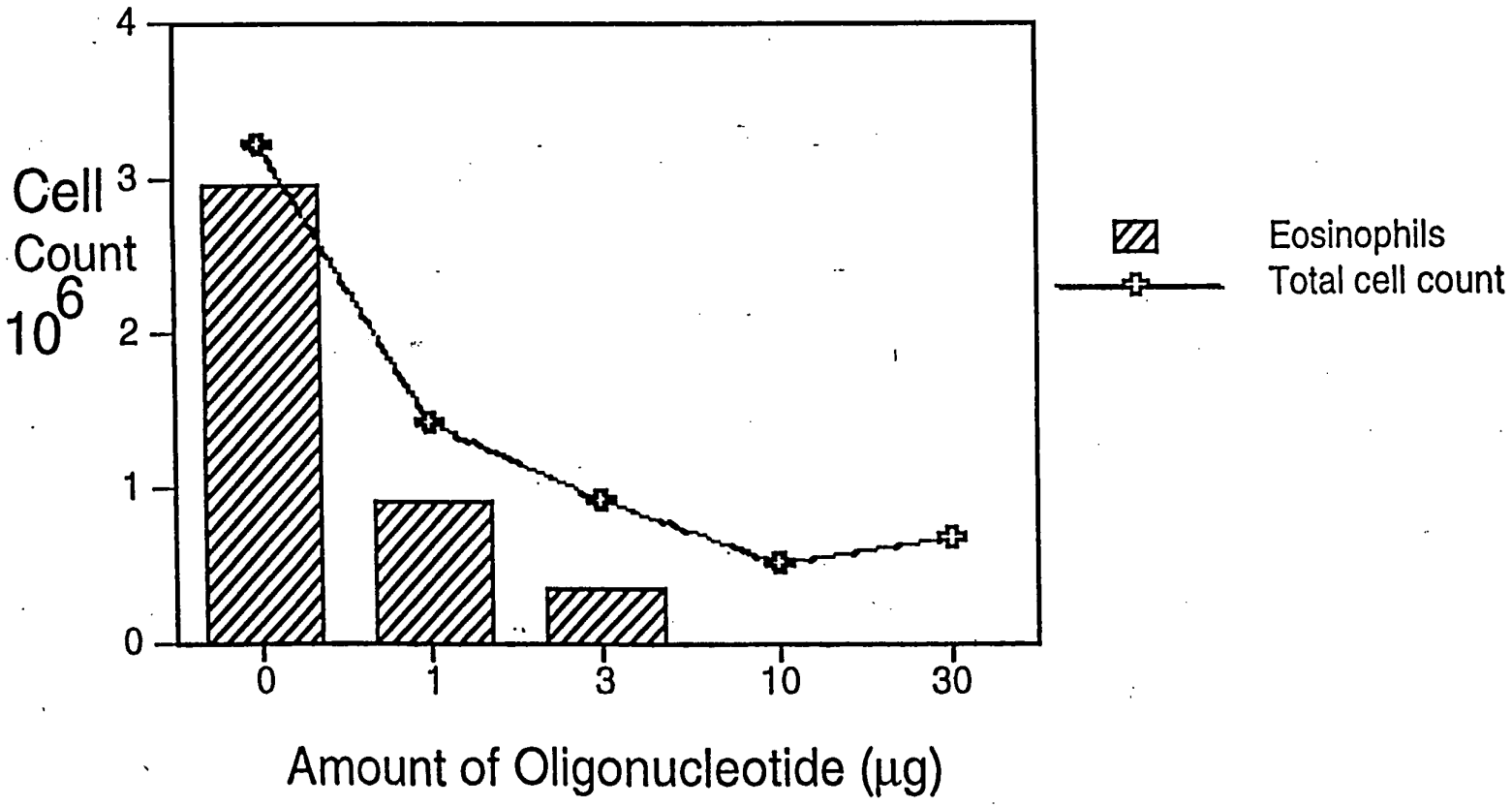


FIGURE 13

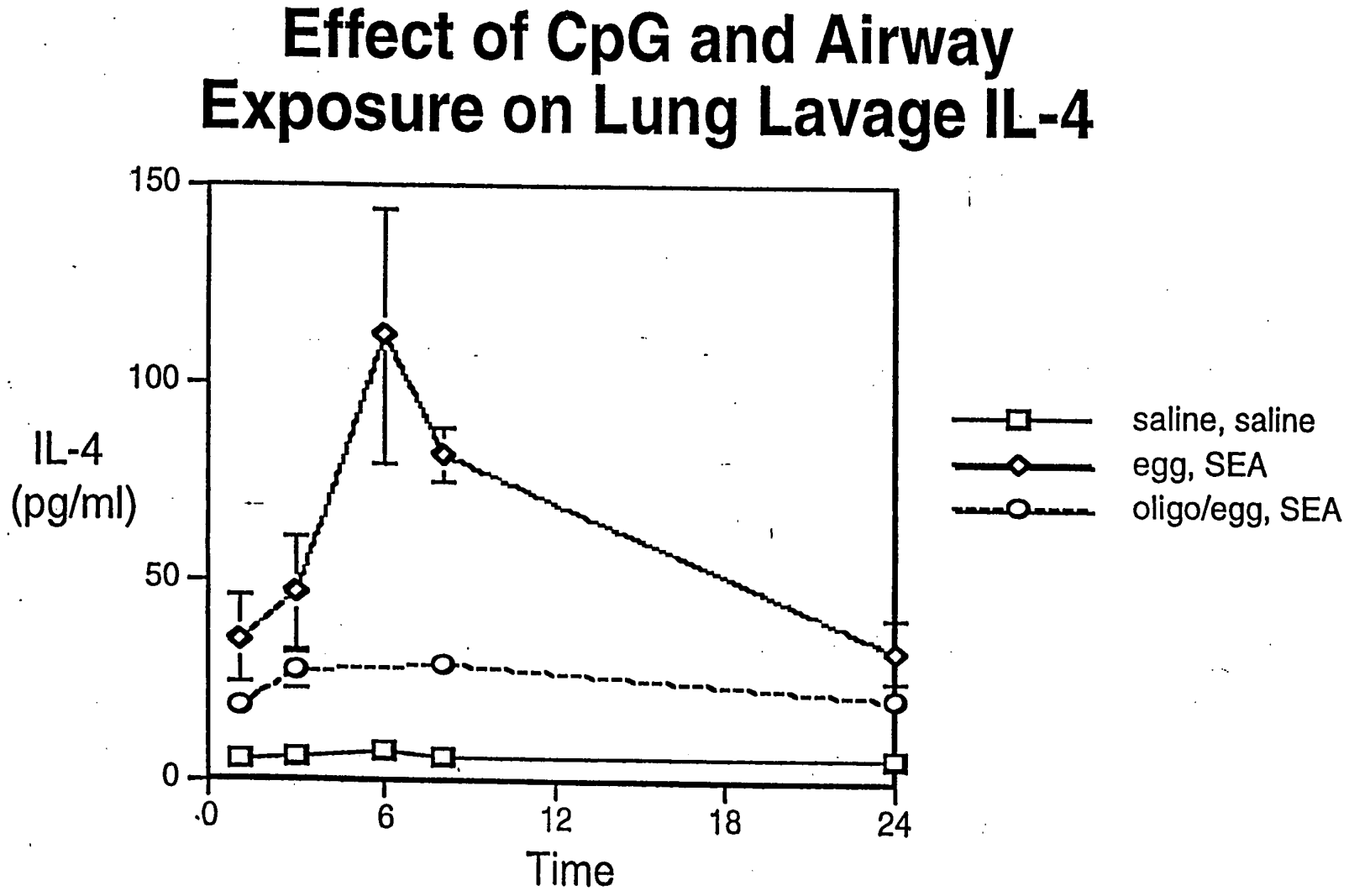

Effect of CpG and Airway Exposure on Lung Lavage IL-4
150
100
50
0
IL-4 (pg/ml)
0
6
12
18
24
Time
saline, saline
egg, SEA
oligo/egg, SEA

FIGURE 14

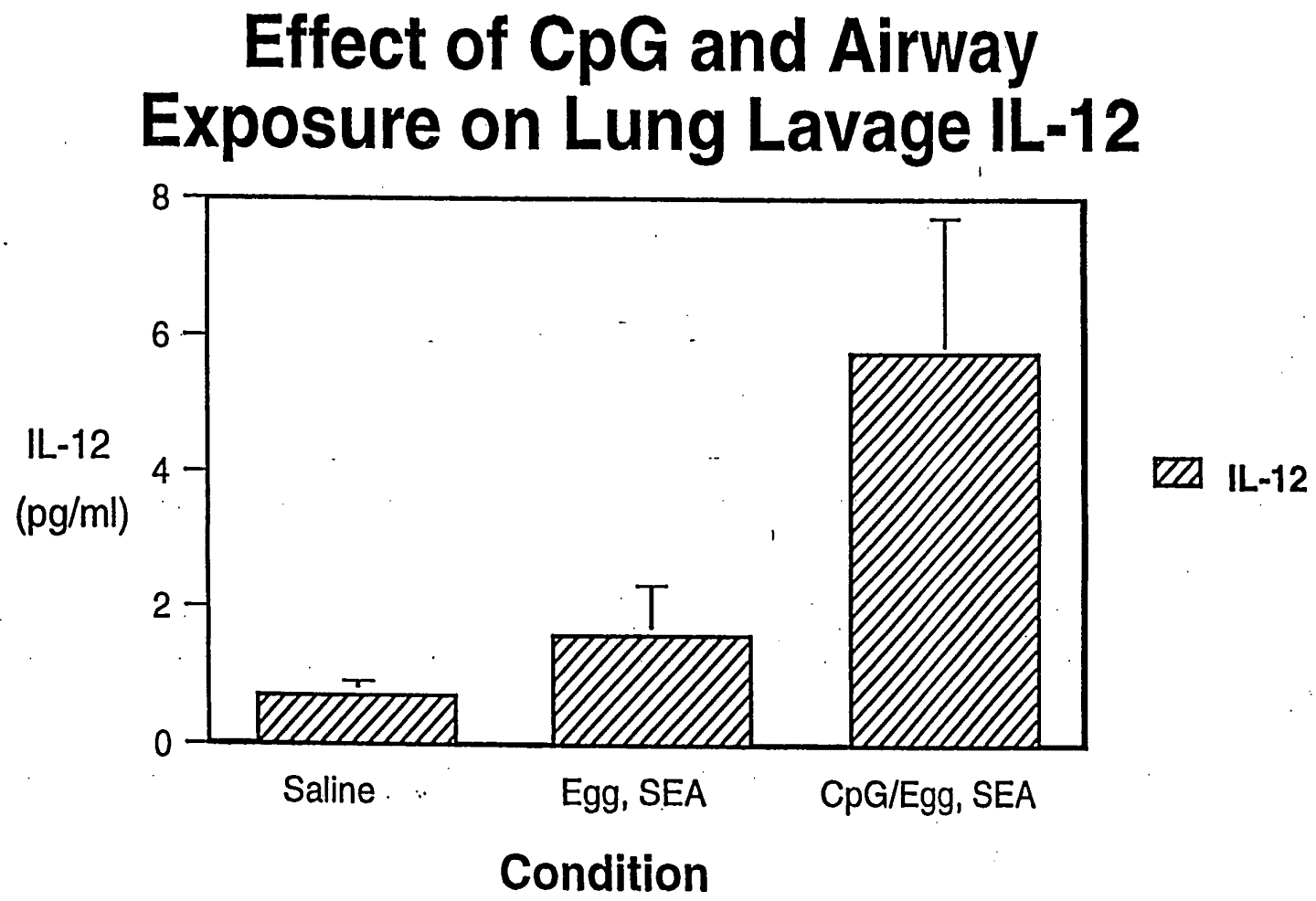
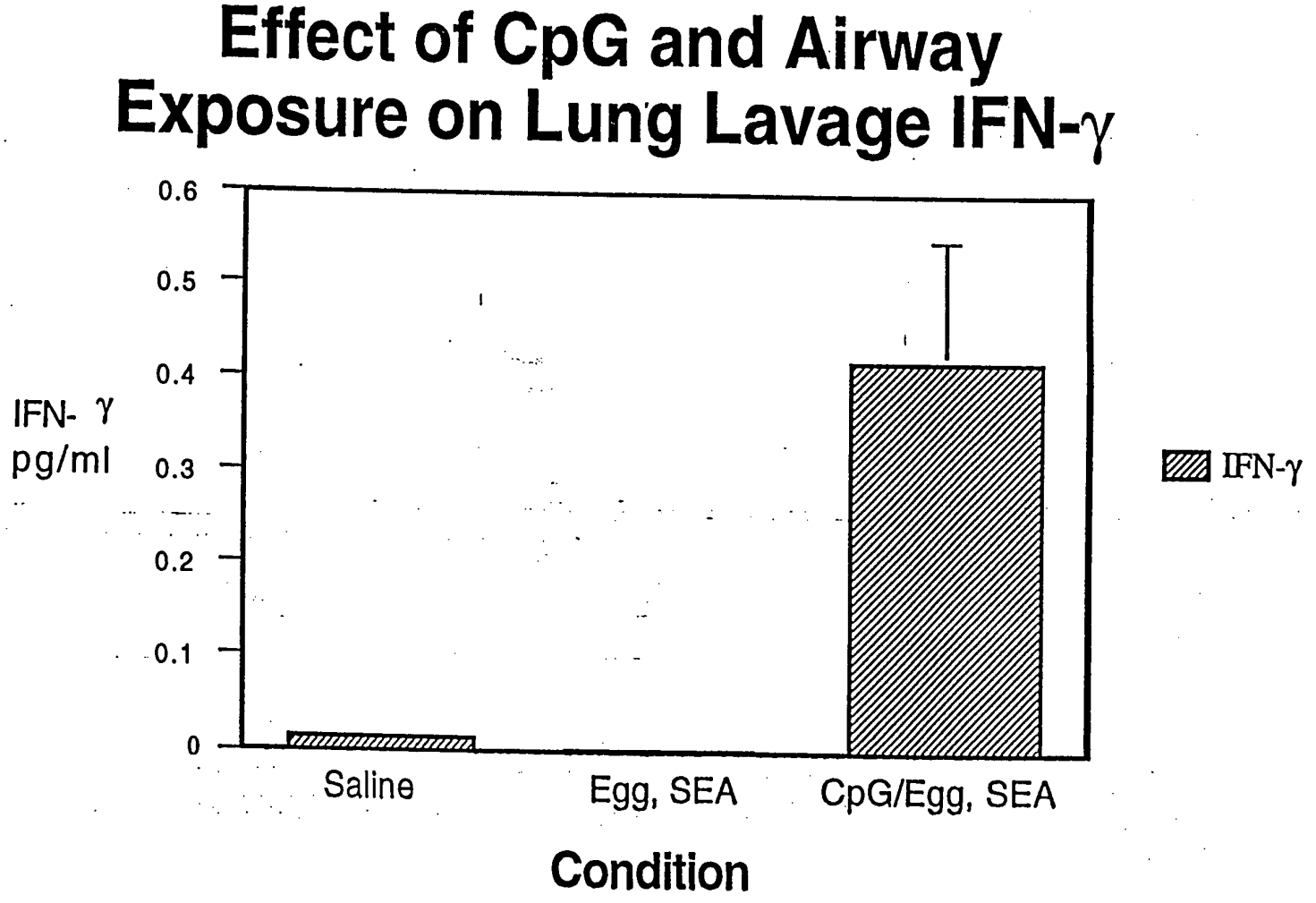

Effect of CpG and Airway Exposure on Lung Lavage IL-12
IL-12 (pg/ml)
8
6
4
2
0
Saline
Egg, SEA
CpG/Egg, SEA
Condition
IL-12

FIGURE 15


Effect of CpG and Airway Exposure on Lung Lavage IFN-γ
IFN- γ pg/ml
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline
Egg, SEA
CpG/Egg, SEA
Condition
IFN-γ